UNITED STATES DISTRICT COURT

DISTRICT OF NEW JERSEY

Janssen Biotech, Inc. Plaintiff, vs. Samsung Bioepis Co., Ltd. Defendant.	Civil Action No.

Plaintiff Janssen Biotech, Inc. ("Janssen") for its Complaint against Defendant Samsung Bioepis Co., Ltd. ("Bioepis" or "Defendant") alleges as follows.

NATURE OF THE ACTION

1. This is an action for patent infringement under 35 U.S.C. § 271(e)(2)(C), which was enacted in 2010 in the part of the Patient Protection and Affordable Care Act known as the Biologics Price Competition and Innovation Act ("BPCIA").

2. This is also an action to enforce the patent dispute resolution provisions of the BPCIA, which Bioepis has refused to follow to date.

3. The BPCIA created an abbreviated regulatory pathway for the approval of biosimilar versions of biological medicines. The BPCIA pathway allows biosimilars makers to avoid the full complement of pre-clinical and clinical studies required for regulatory approval and instead rely on data supporting the safety and efficacy of the innovative biological product which the biosimilar mimics. By taking advantage of the BPCIA regulatory pathway, biosimilars makers can greatly reduce the time and expense of obtaining marketing approval.

4. In order to prevent the new biosimilar pathway from undermining the intellectual property rights of innovators and thereby deterring innovation, the BPCIA also created an intricate and carefully orchestrated set of dispute resolution procedures to facilitate the orderly resolution of patent disputes before a biosimilar product could enter the market.

5. Pursuant to the BPCIA, Bioepis submitted an abbreviated Biologic License Application ("aBLA") seeking permission to market a proposed biosimilar version of Janssen's revolutionary biological medicine Remicade® (infliximab).

6. Bioepis's aBLA was accepted for review by the Food and Drug Administration ("FDA") on May 20, 2016.

7. The FDA approved Bioepis's aBLA on April 21, 2017.

8. The BPCIA's dispute resolution procedure involves a series of information exchanges and good-faith negotiations between the parties before the filing of a patent infringement lawsuit. Bioepis, however, has refused to follow these procedures.

9. Despite making use of the BPCIA's abbreviated regulatory pathway and Janssen's research and development leading to the approval of Remicade®, Bioepis short-circuited the BPCIA's patent dispute resolution process by withholding the information necessary for Janssen to assess infringement. The U.S. Supreme Court is currently evaluating whether a biosimilar maker may lawfully make use of the BPCIA's regulatory pathway while failing to provide its aBLA and manufacturing information to the innovator company so as to allow the innovator company to assess infringement.

10. Bioepis further thwarted the BPCIA patent dispute resolution process by serving what it denominated a "notice of commercial marketing" well in advance of when it was allowed

to do so by law. Under applicable law, a biosimilar applicant cannot serve its notice until after the FDA approves the aBLA, resulting in a licensed biosimilar product.

11. Bioepis has indicated that it will comply with applicable law but has not withdrawn its premature, purported "notice of commercial marketing." Bioepis also served a proper notice of commercial marketing on April 21, 2017, upon FDA approval of its aBLA, in accordance with applicable law. The issue of whether a notice of commercial marketing must be provided after licensing under the BPCIA, as the Court of Appeals for the Federal Circuit has held, is currently before the U.S. Supreme Court.

12. In light of Bioepis's actions as well as the issues before the U.S. Supreme Court, Janssen is asserting, in addition to its claims for violations of the BPCIA, claims for infringement of three patents under 35 U.S.C. § 271(e)(2)(C)(ii) based on Bioepis's submission of the aBLA and its failure to provide its aBLA and manufacturing information to Janssen as set forth under the BPCIA.

PARTIES

13. Janssen Biotech, Inc. is a company organized and existing under the laws of the Commonwealth of Pennsylvania, with a principal place of business in Horsham, Pennsylvania.

14. On information and belief, Samsung Bioepis Co., Ltd. is a company organized and existing under the laws of the Republic of Korea. Samsung Bioepis Co., Ltd. is a biopharmaceutical company that specializes in research and development of biosimilars and biopharmaceuticals. Samsung Bioepis Co., Ltd. markets and distributes such biopharmaceutical products in the United Sates, including through its distributor and commercialization partner Merck & Co., Inc.

JURISDICTION AND VENUE

15. This is an action for violations of 42 U.S.C. § 262(*l*), and patent infringement under the patent laws of the United States, Title 35, United States Code. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331, 1338(a), 2201(a), and 2202.

16. On information and belief, Bioepis has been approved to market and distribute its proposed biosimilar infliximab product throughout the United States, including in New Jersey.

17. On information and belief, Bioepis has entered into a development and commercialization agreement with Merck & Co., Inc. ("Merck") to market and distribute its biosimilar infliximab product in New Jersey. Merck is headquartered in New Jersey.

18. On information and belief, and as Bioepis has asserted, Bioepis intends to "engage in the commercial manufacture, use, and/or sale" of its biosimilar infliximab product, which will be purposefully directed at New Jersey and throughout the United States.

19. On information and belief, and as Bioepis has asserted, Bioepis intends to engage in the commercial manufacture, use, or sale of its biosimilar infliximab product before the expiration of Janssen's patents throughout the United States, including in New Jersey. Indeed, Bioepis has been approved to engage in such commercial manufacture, use and sale.

20. On information and belief, Bioepis's development and commercialization agreement with Merck indicates that Bioepis plans to market its proposed biosimilar infliximab in New Jersey. Whether or not Bioepis itself intends to sell its biosimilar infliximab directly into New Jersey, it has a distributor with which it contracts to market its biosimilar infliximab in New Jersey.

21. Bioepis's intended sales of its biosimilar infliximab before the expiration of Janssen's patents would irreparably injure Janssen in New Jersey by, among other things, displacing its New Jersey sales.

22. Bioepis's submission of its aBLA to the FDA evinces its intent to subject itself to the jurisdiction of the courts where the drug that is the subject of the aBLA will be sold, including New Jersey.

23. This Court's exercise of personal jurisdiction over Bioepis is fair and reasonable. Bioepis is not burdened by litigating this suit in New Jersey. New Jersey has an interest in providing a forum to resolve BPCIA litigation, including this case, because this litigation involves products that will be sold in New Jersey by a New Jersey-based company and injury to Janssen in New Jersey. This Court's exercise of jurisdiction serves the interests of Janssen and the judicial system in efficient resolution of litigation.

24. In the alternative, this Court's exercise of personal jurisdiction over Bioepis is also proper pursuant to Federal Rule of Civil Procedure 4.

25. Under Rule 4(k)(2), for a claim arising under federal law, jurisdiction in any federal court is proper where a defendant is (1) not subject to general jurisdiction in any state and (2) exercise of jurisdiction is consistent with the United States Constitution and laws.

26. Bioepis has availed itself of the laws of the United States by, among other things, seeking and receiving approval for its biosimilar.

27. Litigating in the District of New Jersey would not burden Bioepis unduly. The United States has a substantial interest in adjudicating the dispute and enforcing its patent laws. Janssen has a substantial interest in obtaining convenient and effective relief for violations of its property interests. And the states also have a shared interest in furthering the fundamental substantive policy of the United States with respect to its intellectual property laws.

28. Venue is proper in this judicial district under 28 U.S.C. §§ 1391(b), (c) and/or 1400(b).

REMICADE® (INFLIXIMAB)

29. Janssen is a pioneer and leader in the development of biologic drugs. Janssen's biologic drug Remicade® was one of the first drugs of its kind sold in the United States for treatment of chronic disease.

30. Remicade® is a monoclonal antibody that binds to and neutralizes a substance in our bodies called TNFα. TNFα is an important player in our immune systems but, if it is over-produced, it can cause chronic disease.

31. Although the antibody had promising in vitro properties, given its complex structure and mechanism of operation it required extensive pre-clinical and clinical development before it could become a useful medicine for human beings.

32. From the time the infliximab antibody was first discovered, it took nearly a decade for Remicade® to be approved for sale in the United States. During that time, Janssen's predecessor Centocor conducted dozens of clinical trials and spent tens of millions of dollars, with no guarantee of success.

33. Remicade® was first approved for the U.S. market in 1998. The first indication, or use, for which Remicade® was approved was the treatment of Crohn's disease, an inflammatory bowel disease that causes inflammation of the lining of the digestive tract. Remicade® was the first biological therapy approved for Crohn's disease in the United States.

34. After Remicade® entered the market, Centocor continued to pursue extensive clinical development efforts for the drug. These efforts led to the discovery that Remicade® is safe and effective for a number of additional diseases and indications other than Crohn's disease.

35. Janssen's extensive development efforts have led to 16 FDA approvals for Remicade®, including indications for use in the treatment of Crohn's disease (1998), rheumatoid arthritis (1999), ankylosing spondylitis, a chronic inflammatory disease of the axial skeleton

(2004), psoriatic arthritis (2005), and ulcerative colitis, an inflammatory bowel disease (2006). Remicade® has changed the standard of care for the treatment of these diseases.

36. In total, Janssen has sponsored more than 170 clinical trials for Remicade®. Janssen has spent hundreds of millions of dollars in research and development of the drug.

37. Remicade® had been used to treat and improve the lives of more than 2.2 million patients suffering from chronic disease.

JANSSEN'S CELL CULTURE MEDIA AND PURIFICATION PATENTS

38. Janssen asserts three of its patents for growing and purifying biologics, including infliximab, in this action.

The Chemical Cell Growth Media Patents (the '083 Patent and the '056 Patent)

39. On October 6, 2009, the U.S. Patent and Trademark Office ("PTO") issued U.S. Patent No. 7,598,083 (the "'083 patent"), entitled "Chemically Defined Media Compositions." A true and correct copy of the '083 patent is attached as Exhibit A.

40. On May 31, 2005, the PTO issued U.S. Patent No. 6,900,056 (the "'056 patent), entitled "Chemically Defined Medium for Cultured Mammalian Cells." A true and correct copy of the'056 patent is attached as Exhibit B.

41. Janssen owns the '083 and '056 patents, which cover cell growth media for use in growing biological products, including infliximab.

42. The '083 patent will expire on February 7, 2027.

43. The '056 patent will expire on October 5, 2022.

The Purification Patent (the ’600 patent)

44. On August 10, 2004, the PTO issued U.S. Patent no. 6,773,600 (the “’600 patent”), entitled “Use of Clathrate Modifier, To Promote Passage of Proteins During Nanofiltration.” A true and correct copy of the ’600 patent is attached as Exhibit C.

45. Janssen owns the ’600 patent, which covers novel methods of purifying biological products such as infliximab so that they are suitable for use in human medicines.

46. The ’600 patent will expire on June 4, 2023.

BIOLOGICS, BIOSIMILARS, AND THE BPCIA

Biologics

47. Biological medicines, or biologics, are complex biological molecules that need to be grown in living cultures rather than chemically synthesized, as are the more familiar pharmaceutical products known as chemical or small-molecule drugs. Because the biologic manufacturing process is complex and uses living organisms, the structural features of a biologic drug can vary based on the precise manner in which the drug is made. Unlike small-molecule drugs, moreover, biological molecules generally cannot be completely characterized.

48. Because of the differences between biological and small-molecule drugs, biological and small-molecule pharmaceutical products are approved for sale in the United States through different regulatory pathways. Whereas small-molecule drugs are approved based on the submission of a New Drug Application (“NDA”) (*see* 21 U.S.C. § 355), biological products are assessed pursuant to a Biological License Application (“BLA”) (*see* 42 U.S.C. § 262(a)).

The BPCIA Pathway for Biosimilar Approval

49. Although Congress created an abbreviated regulatory pathway for the approval of generic small-molecule drugs in the Hatch-Waxman Act of 1984, no abbreviated pathway for approval of follow-on biologics products existed until the enactment of the BPCIA, as part of the

Patient Protection and Affordable Care Act, in 2010. Before the enactment of the BPCIA, the only way to obtain U.S. approval of a biological product was through an original BLA supported by a full complement of pre-clinical and clinical data.

50. The BPCIA creates an abbreviated approval pathway for FDA licensure of biological products upon a determination that the biological product is “biosimilar” to a previously licensed “reference product.” 42 U.S.C. § 262(k). The BPCIA defines a “biosimilar” as a biological product that is (1) “highly similar to the reference product notwithstanding minor differences in clinically inactive components”; and (2) has “no clinically meaningful differences” with “the reference product in terms of the safety, purity, and potency of the product.” 42 U.S.C. §§ 262(i)(2)(A), (B). The BPCIA defines a “reference product” to be a “single biological product licensed under subsection (a) against which a biological product is evaluated in an application submitted under subsection (k).” 42 U.S.C. § 262(i)(4).

51. Under the BPCIA, biosimilar applicants are permitted to make use of FDA’s prior determinations as to the safety, purity, and potency of a reference product that was already approved by FDA. In particular, a biosimilar applicant must identify a single reference product that has already been approved by FDA and submit to FDA “publicly-available information regarding the Secretary’s previous determination that the reference product is safe, pure, and potent.” 42 U.S.C. § 262(k)(2)(A)(iii)(I). Consequently, the § 262(k) pathway created by the BPCIA allows the biosimilar applicant to reduce the time, expense, and risks of research and development, avoid the full complement of pre-clinical and clinical testing required for an original product, and gain licensure to commercialize its biological product in the market as a biosimilar sooner and more cheaply than it could have done through the submission of an original BLA.

The BPCIA's Patent Dispute Resolution Procedures

52. As Congress expressly indicated, the purpose of the BPCIA is to establish "a biosimilars pathway balancing innovation and consumer interests." Biologics Price Competition and Innovation Act of 2009, Pub. L. No. 111-148, § 7001(b), 124 Stat. 119, 804 (2010).

53. To further this goal, Congress created a set of procedures for addressing patent disputes relating to prospective biosimilar drugs. These procedures are set forth in 42 U.S.C. § 262(*l*) and in corresponding amendments to the patent infringement statute, 35 U.S.C. § 271. The procedures are intended to ensure that the maker of an innovative biological product that is the subject of a biosimilar application will have sufficient time and opportunity to enforce its patent rights before a biosimilar product enters the United States market. The BPCIA's patent dispute resolution procedures are also intended to ensure that disputes over patent rights will take place in an orderly fashion, with the least possible uncertainty, brinksmanship, and burden on the parties and the courts.

54. The BPCIA patent dispute resolution procedures set forth a series of specific steps before any patent action is filed.

55. The BPCIA dispute resolution process begins when a biosimilar application is accepted for review by FDA. Within twenty days thereafter, the biosimilar applicant "shall provide" the reference sponsor with confidential access to "a copy of the application submitted . . . under subsection (k), and such other information that describes the process or processes used to manufacture the biological product that is the subject of such application." 42 U.S.C. § 262(*l*)(2)(A). This step initiates a series of pre-litigation exchanges of information and positions so that the parties are able to engage in good-faith negotiations regarding what patents should be litigated prior to the approval of the biosimilar product. *See* 42 U.S.C. § 262(*l*)(2)–(*l*)(6).

56. The BPCIA's requirement that manufacturing information be provided reflects the complexity of manufacturing processes for biologics and their importance to innovation in the field. To ensure that full application and manufacturing information be made available without prejudice or delay, the BPCIA sets forth a detailed set of confidential access provisions governing the reference product sponsor's use of the required information. 42 U.S.C. § 262(*l*)(1).

57. The next step in the statutory process is 42 U.S.C. § 262(*l*)(3)(A). This section provides that within 60 days after receiving the information set forth in section 262(*l*)(2), the reference product sponsor "shall provide" the biosimilar applicant a list of patents for which the reference product sponsor "believes a claim of patent infringement could reasonably be asserted" against the proposed biosimilar product or the uses or manufacture of such product. 42 U.S.C. § 262(*l*)(3)(A)(i). The reference product sponsor also states whether it is willing to license any of these patents. 42 U.S.C. § 262(*l*)(3)(A)(ii).

58. The next statutory provision, section 262(*l*)(3)(B), states that within 60 days the biosimilar applicant "shall provide" a "detailed statement" of its non-infringement, invalidity, and unenforceability defenses with respect to the listed patents, or a statement that the applicant "does not intend to bring commercial marketing of the biological product before the date that such patent expires." 42 U.S.C. § 262(*l*)(3)(B)(ii).

59. The next statutory provision, section 262(*l*)(3)(C), states that within 60 days the reference product sponsor "shall provide" a "detailed statement" of its infringement positions and "a response to the statement concerning validity and enforceability provided" by the biosimilar applicant. 42 U.S.C. § 262(*l*)(3)(C).

60. After the exchange of detailed statements, the statute provides that the parties "shall engage in good faith negotiations" to agree on patents that will be subject to an action for patent infringement prior to the approval of the biosimilar application. 42 U.S.C. § 262(*l*)(4)(A).

61. If the parties agree on the patents that will be subject to an immediate action for infringement, then the reference product sponsor "shall bring an action for patent infringement" within thirty days of the agreement. 42 U.S.C. § 262(*l*)(6)(A). If the parties fail to reach agreement, they proceed to a further exchange process that will identify one or more patents for immediate litigation. 42 U.S.C. § 262(*l*)(4)(B) & (*l*)(5). As in the case of agreement, the reference product sponsor "shall bring an action for patent infringement" within thirty days after patents are selected for litigation through this process. 42 U.S.C. § 262(*l*)(6)(B).

Notice of Commercial Marketing

62. In addition to the pre-litigation procedures described above, the BPCIA addresses litigation regarding a "biological product licensed under subsection (k)"—*i.e.*, a biosimilar product that has been approved for marketing. The BPCIA requires the biosimilar maker to provide "notice to the reference product sponsor not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)." 42 U.S.C. § 262(*l*)(8)(A).

63. Upon receipt of a notice of commercial marketing, the reference product sponsor may move for a preliminary injunction on patents that the sponsor identified as potentially infringed under section 262(*l*)(3)(A) of the pre-litigation dispute resolution procedures, but which the parties have not selected for litigation pursuant to these procedures. 42 U.S.C. § 262(*1*)(8)(B).

64. In addition, the notice of commercial marketing permits the reference product sponsor to bring a declaratory judgment action with respect to such patents that have been identified but not selected for immediate litigation. 42 U.S.C. § 262(*1*)(9)(A). Before the notice of commercial marketing, such declaratory judgments are prohibited. *Id.*

BIOEPIS'S BIOSIMILAR PRODUCT

65. On information and belief, Bioepis has undertaken the development of a biosimilar to Janssen's Remicade® infliximab product.

66. On information and belief, in 2013, Bioepis entered into an agreement with Merck pursuant to which Merck obtained the rights to market biosimilar infliximab in the United States. The proposed biosimilar infliximab product to be marketed by Bioepis is referred to as SB2 or Renflexis®. It is also marketed in Europe as Flixabi®.

67. On information and belief, the FDA accepted Bioepis's aBLA for this proposed biosimilar product on or about May 20, 2016.

68. On information and belief, the FDA approved Bioepis's proposed biosimilar product on April 21, 2017, for Crohn's disease, pediatric Crohn's disease, ulcerative colitis, rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and plaque psoriasis.

BIOEPIS'S EFFORTS TO AVOID THE BPCIA'S PATENT DISPUTE RESOLUTION PROCEDURES

69. From the time it began the process of seeking approval for its proposed biosimilar product, Bioepis has sought to avoid the mandatory patent dispute resolution procedures of the BPCIA. Bioepis has elected to short-circuit the statutory process by withholding its aBLA and manufacturing information, by refusing to participate to date in subsequent statutory procedures, and by serving a premature "notice of commercial marketing."

70. As set forth above, the BPCIA's dispute resolution procedures are triggered by FDA's acceptance of a biosimilar application for review. 42 U.S.C. § 262(*l*)(2)(A).

71. After the FDA accepted Bioepis's application for review on May 20, 2016, it sent Janssen a letter dated May 26, 2016, attached as Exhibit D.

72. In its letter, Bioepis notified Janssen that its application had been accepted by the FDA for review pursuant to the BPCIA.

73. Bioepis wrote that it was refusing to comply with the BPCIA's exchange information procedures, stating that it would "not provide Janssen Biotech, Inc. with a copy of BLA No. 761054 or any information that describes the process or processes used to manufacture the biological product that is the subject of BLA No. 761054."

74. Bioepis invited Janssen to bring suit against it on any patent that "claims the biological product or its use or its manufacture" while withholding all information necessary to evaluate infringement.

75. Lastly, though Bioepis's product had not been approved by the FDA, Bioepis's May 26, 2016 letter purported to provide notice that it would "commence commercial marketing . . . as soon as possible under applicable law after the FDA's approval to do so but no earlier than 180 days from receipt of this notice by Janssen Biotech, Inc."

76. On January 12, 2017, Janssen asked Bioepis to commit not to commercially market its proposed biosimilar until 180 days after FDA approval, as required by law.

77. Bioepis responded by letter of January 22, 2017, attached as Exhibit E.

78. The January 22, 2017 letter stated that Bioepis continued to regard its May 26, 2016, letter as constituting effective notice of commercial marketing. Bioepis acknowledged that under controlling law its early "notice" was ineffective but stated that the effectiveness might

change depending on the outcome of the U.S. Supreme Court's forthcoming decision in *Amgen v. Sandoz* (No. 15-1159). Bioepis concluded by stating that it would "assert its rights in accordance with the decision of the U.S. Supreme Court and will provide Janssen with an additional commercial marketing notice at the time of FDA licensure if necessary."

79. On April 21, 2017, upon FDA's approval of its biosimilar product, Bioepis sent an additional letter, attached as Exhibit F.

80. In the April 21, 2017 letter, Bioepis stated again that the May 26, 2016 letter was an effective notice of commercial marketing and that it is "authorized by the FDA to commercially market" its biosimilar and it would "commercially market" the biosimilar "as soon as possible under applicable law."

81. In accordance with controlling law, Bioepis stated that the April 21, 2017 letter should also be treated as a notice of commercial marketing, in the event that the May 26, 2016, letter is "held to be void or otherwise ineffective."

82. As of its letter of April 21, 2017, Bioepis has refused to participate in any of the BPCIA's patent dispute resolution procedures, and Bioepis has made it impossible for Janssen to assess which of Janssen's patents are infringed.

83. Given Bioepis's refusal to comply with the provisions of the BPCIA so as to allow Janssen to assess infringement of its patents and protect its intellectual property rights, Janssen has filed this Complaint to protect its interests and to enforce its patents and the statutory provisions which Bioepis seeks to bypass.

Bioepis's Premature Notice of Commercial Marketing

84. On May 26, 2016, in the same letter in which Bioepis refused to provide its aBLA and manufacturing information, Bioepis stated (prematurely) that it was providing a notice of

commercial marketing, purportedly pursuant to 42 U.S.C. § 262(*l*)(8)(A). Bioepis asserted that it would begin commercial marketing of its proposed biosimilar product "as soon as possible" after the FDA's approval and as early as "180 days from the receipt of this notice," i.e., as early as November 22, 2016.

85. The BPCIA includes a clear condition precedent to providing a notice of commercial marketing. The statutorily required notice is "of the first commercial marketing of the biological product licensed under subsection (k)." 42 U.S.C. § 262(*l*)(8)(A). The grant of a license under subsection (k) is a statutory prerequisite to providing a notice of commercial marketing.

86. As Bioepis is aware, this was precisely the holding of the Federal Circuit in *Amgen, Inc. v. Sandoz, Inc.*, 794 F.3d 1347 (Fed. Cir. 2015) and *Amgen, Inc. v. Apotex Inc.*, 827 F.3d 1052 (Fed. Cir. 2016). In *Amgen v. Sandoz* the Federal Circuit held that a biosimilar application "may only give effective notice of commercial marketing after the FDA has licensed its product." *Sandoz*, 794 F.3d at 1357. The Federal Circuit reaffirmed that holding in *Amgen v. Apotex*, explaining that "the notice starting the 180-day clock must follow, not precede, the licensure." *Apotex*, 827 F.3d at 1056.

87. Bioepis had not, at the time of its May 26, 2016 letter, received a license to market its proposed biosimilar product under subsection (k). As a result, Bioepis's proposed product was not a "biological product licensed under subsection (k)" and could not be the subject of a valid notice of commercial marketing pursuant to the BPCIA.

88. The purpose of the notice of commercial marketing provision is to provide the parties and the Court with sufficient time (180 days) to resolve any disputes that need to be resolved before commercial launch of a biosimilar product. If Bioepis is allowed to proceed

based on its invalid notice of commercial marketing, the 180-day period would have already run, during a time when the precise nature of the dispute between the parties, and even the need for litigation on certain patents, had not yet crystallized.

89. On January 12, 2017, Janssen asked Bioepis to withdraw its premature notice of commercial marketing.

90. On January 22, 2017, Bioepis refused to withdraw the notice of commercial marketing, although it confirmed that it would comply with applicable law.

91. On April 21, 2017, Bioepis provided Janssen with a post-licensure notice of commercial marketing but said it would rely on its prior notice if the law changes.

COUNT 1: VIOLATION OF MANDATORY PROCEDURES UNDER 42 U.S.C. § 262(*l*)(2)

92. Janssen incorporates by reference paragraphs 1-91 as if fully set forth herein.

93. This claim arises under the BPCIA, 42 U.S.C. § 262(*l*)(2), and the Declaratory Judgment Act, 28 U.S.C. §§ 2201(a) & 2202.

94. The BPCIA, 42 U.S.C. § 262(*l*)(2), provides procedures to resolve patent disputes related to the filing of an aBLA under 42 U.S.C. § 262(k).

95. Bioepis has failed to comply with the requirements of the BPCIA. Bioepis's failure to follow the procedures set forth in the BPCIA has injured Janssen by depriving it of the procedural protections of the statute and by subjecting it to the burden of unnecessary litigation.

96. Contrary to 42 U.S.C. § 262(*l*)(2)(A), Bioepis did not provide Janssen with a copy of the aBLA "and such other information that describes the process or processes used to manufacture the biological product that is the subject of such application." Indeed, Bioepis expressly refused to do so. By refusing to provide the information set forth under the BPCIA, Bioepis has made it impossible for Janssen to assess infringement of its patents. Bioepis has

benefited from the BPCIA's regulatory pathway and Janssen's years of research and development while withholding information critical for Janssen to protect its patent rights.

97. Bioepis's failure to follow the BPCIA's procedures, individually and collectively, has caused and will cause Janssen injury, including irreparable harm for which Janssen has no adequate remedy at law, and will continue unless the statutory requirements are declared and enforced by this Court.

98. Under current law, the Federal Circuit has not interpreted 42 U.S.C. § 262(*l*)(2)(A) to be mandatory. *See Amgen, Inc. v. Sandoz, Inc.*, 794 F.3d 1347, 1357 (Fed. Cir. 2015).

99. However, on January 13, 2017, the Supreme Court granted *certiorari* on this precise issue in *Amgen, Inc. v. Sandoz, Inc.* (U.S. Jan. 13, 2017) (No. 15-1195).

100. As such, Janssen includes this Count 1 as the Supreme Court may hold that biosimilar makers that make use of the BPCIA's regulatory procedures also must follow its patent dispute resolution procedures, the first step of which is 42 U.S.C. § 262(*l*)(2)(A).

COUNT 2: VIOLATION OF MANDATORY PROCEDURES UNDER 42 U.S.C. § 262(*l*)

101. Janssen incorporates by reference paragraphs 1-100 as if fully set forth herein.

102. The BPCIA provides that a biosimilar applicant "shall provide notice to the reference product sponsor not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)." 42 U.S.C. § 262(*l*)(8)(A).

103. This requirement has been held by the Federal Circuit to be mandatory. *See Amgen, Inc. v. Apotex Inc.*, 827 F.3d 1052, 1056 (Fed. Cir. 2016), *cert. denied*, 85 U.S.L.W. 3287 (U.S. Dec. 12, 2016) (No. 16-332) ("[T]he notice starting the 180-day clock must follow, not precede, the licensure."); *Amgen, Inc. v. Sandoz, Inc.*, 794 F.3d 1347, 1357 (Fed. Cir. 2015)

("[A] subsection (k) applicant may only give effective notice of commercial marketing after the FDA has licensed its product.").

104. Bioepis has provided two letters purportedly providing notice of commercial marketing, the first of which is not effective. Bioepis has refused to withdraw the ineffective notice but states that it will comply with applicable law.

105. If Bioepis were to rely on an ineffective notice or not comply with the law, its violation of the notice of commercial marketing provision would cause Janssen injury, including irreparable harm for which Janssen has no adequate remedy at law, and will continue unless the statutory requirement is declared and enforced by this Court.

COUNT 3: INFRINGEMENT OF THE '083 PATENT

106. Janssen incorporates by reference paragraphs 1–105 as if fully set forth herein.

107. On information and belief, Bioepis has been aware of the '083 patent since a time before Bioepis filed its aBLA.

108. Bioepis's submission of its aBLA was an act of infringement of the '083 patent under 35 U.S.C. § 271(e)(2)(C)(ii), literally or under the doctrine of equivalents.

109. Upon information and belief, Bioepis's infringement of the '083 patent would be willful and would make this case exceptional entitling Janssen to attorneys' fees.

110. Unless Bioepis is enjoined from infringing the '083 patent, Janssen will suffer irreparable injury for which damages are an inadequate remedy.

COUNT 4: INFRINGEMENT OF THE '056 PATENT

111. Janssen incorporates by reference paragraphs 1–110 as if fully set forth herein.

112. On information and belief, Bioepis has been aware of the '056 patent since a time before Bioepis filed its aBLA.

113. Bioepis's submission of its aBLA was an act of infringement of the '056 patent under 35 U.S.C. § 271(e)(2)(C)(ii), literally or under the doctrine of equivalents.

114. Upon information and belief, Bioepis's infringement of the '056 patent would be willful and would make this case exceptional entitling Janssen to attorneys' fees.

115. Unless Bioepis is enjoined from infringing the '056 patent, Janssen will suffer irreparable injury for which damages are an inadequate remedy.

COUNT 5: INFRINGEMENT OF THE '600 PATENT

116. Janssen incorporates by reference paragraphs 1–115 as if fully set forth herein.

117. On information and belief, Bioepis has been aware of the '600 patent since a time before Bioepis filed its aBLA.

118. Bioepis's submission of its aBLA was an act of infringement of the '600 patent under 35 U.S.C. § 271(e)(2)(C)(ii), literally or under the doctrine of equivalents.

119. Upon information and belief, Bioepis's infringement of the '600 patent would be willful and would make this case exceptional entitling Janssen to attorneys' fees.

120. Unless Bioepis is enjoined from infringing the '600 patent, Janssen will suffer irreparable injury for which damages are an inadequate remedy.

PRAYER FOR RELIEF

WHEREFORE, Janssen respectfully requests that this Court enter judgment in its favor against Bioepis and grant the following relief:

(a) a declaration that Bioepis has failed to comply with the requirements of the BPCIA patent dispute resolution process, including 42 U.S.C. § 262(*l*)(2)(A);

(b) an order compelling Bioepis to comply with the BPCIA patent dispute resolution process set forth in 42 U.S.C. § 262(*l*);

(c) a declaration that the "notice of commercial marketing" provided by Bioepis on May 26, 2016 is not an effective "notice of commercial marketing" within the meaning of 42 U.S.C. § 262(*l*)(8)(A) and that Bioepis may not begin the commercial marketing of its biosimilar to Janssen's Remicade® infliximab product until at least 180 days after a proper notice pursuant to 42 U.S.C. § 262(*l*)(8)(A);

(d) preliminary and/or permanent equitable relief, including but not limited to a preliminary and permanent injunction that enjoins Bioepis, its officers, partners, agents, servants, employees, parents, subsidiaries, divisions, affiliate corporations, other related business entities and all other persons acting in concert, participation, or in privity with them and/or their successors or assigns, from any commercial manufacture, use, offer to sell or sale within the United States, of Bioepis's biosimilar to Janssen's Remicade® infliximab product, until 180 days after a proper notice pursuant to 42 U.S.C. § 262(*l*)(8)(A);

(e) a judgment that Bioepis has infringed under 35 U.S.C. § 271(e)(2)(C)(ii):

(1) the '083 patent;

(2) the '056 patent; and

(3) the '600 patent;

(f) a judgment declaring that the making, using, selling, offering to sell, or importing of the biosimilar to Janssen's Remicade®

infliximab product described in aBLA No. 761054 would constitute infringement of:

(1) the ’083 patent;

(2) the ’056 patent; and

(3) the ’600 patent;

(g) preliminary and/or permanent equitable relief, including but not limited to a preliminary and/or permanent injunction that enjoins Bioepis, its officers, partners, agents, servants, employees, parents, subsidiaries, divisions, affiliate corporations, other related business entities and all other persons acting in concert, participation, or in privity with them and/or their successors or assigns, from any commercial manufacture, use, offer to sell or sale within the United States, or importation into the United Sates, of any product that infringes, or the use or manufacture of which infringes:

(1) the ’083 patent;

(2) the ’056 patent; or

(3) the ’600 patent;

(h) an order compelling Bioepis to compensate Janssen for and awarding damages incurred as a result of Bioepis’s actions or inactions;

(i) a declaration that this is an exceptional case and an award to Janssen of its attorneys’ fees, costs and expenses pursuant to 35 U.S.C. § 271(e)(4) and 35 U.S.C. § 285; and

(j) such other relief as this Court may deem just and proper.

Dated: May 17, 2017

By: */s Michelle M. Bufano*
Michelle M. Bufano
PATTERSON BELKNAP WEBB &
TYLER LLP
1133 Avenue of the Americas
New York, NY 10036-6710

Gregory L. Diskant
Irena Royzman
Aron Fischer
PATTERSON BELKNAP WEBB & TYLER LLP
1133 Avenue of the Americas
New York, NY 10036-6710

CERTIFICATION PURSUANT TO L. CIV. R. 11.2

Pursuant to Local Civil Rule 11.2, I hereby certify that the matter in controversy is not the subject of any other action pending in any court, or of any pending arbitration or administrative proceeding. This action alleges infringement of patents that were asserted in consolidated actions *Janssen Biotech, Inc. et al. v. Celltrion Healthcare Co., Ltd. et al.*, No. 15-cv-10698 (D. Mass. filed Mar. 6, 2015).

By: */s Michelle M. Bufano*
Michelle M. Bufano
PATTERSON BELKNAP WEBB &
TYLER LLP

EXHIBIT A

US007598083B2

(12) **United States Patent**
Epstein et al.

(10) **Patent No.: US 7,598,083 B2**
(45) **Date of Patent: Oct. 6, 2009**

(54) **CHEMICALLY DEFINED MEDIA COMPOSITIONS**

(75) Inventors: **David Epstein**, Philadelphia, PA (US); **Roger Monsell**, Willistown, PA (US); **Joseph Horwitz**, Swarthmore, PA (US); **Susan Lenk**, Devon, PA (US); **Sadettin Ozturk**, Paoli, PA (US); **Christopher Marsh**, Audubon, PA (US)

(73) Assignee: **Centocor, Inc.**, Malvern, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 468 days.

(21) Appl. No.: **11/260,788**

(22) Filed: **Oct. 27, 2005**

(65) **Prior Publication Data**

US 2006/0094113 A1 May 4, 2006

Related U.S. Application Data

(60) Provisional application No. 60/623,718, filed on Oct. 29, 2004.

(51) **Int. Cl.**
C12N 5/00 (2006.01)
C12N 5/02 (2006.01)

(52) **U.S. Cl.** **435/404**; 435/325

(58) **Field of Classification Search** .... None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,282,326	A	8/1981	Moldenhauer et al.
4,533,637	A	8/1985	Yamane et al.
4,657,866	A	4/1987	Kumar et al.
4,767,704	A	8/1988	Cleveland et al.
5,045,468	A	9/1991	Darfler et al.
5,393,668	A	2/1995	Cinatl et al.
6,048,728	A	4/2000	Inlow et al.
6,146,873	A	11/2000	Kistner et al.
6,171,825	B1	1/2001	Chan et al.
6,358,703	B1	3/2002	Cho et al.
6,475,725	B1	11/2002	Reiter et al.
2002/0115152	A1	8/2002	Cho et al.
2002/0182679	A1	12/2002	Reiter et al.
2003/0203448	A1	10/2003	Reiter et al.

FOREIGN PATENT DOCUMENTS

EP	0485689		10/2001
WO	WO 03/00423	A1	1/1993
WO	WO 98/08934	A1	3/1998
WO	WO 03/046162	A2	6/2003

OTHER PUBLICATIONS

Kovár, J and Franěk, F, Serum-free Medium for Hybridoma and Parental Myeloma Cell Cultivation: a Novel Composition of Growth-supporting Substances, *Immunol. Lett.*, vol. 7, (1984), pp. 339-345.

Franěk, F., "Starvation-induced Programmed Death of Hybridoma Cells: Prevention by Amino Acid Mixtures," *Biotechnol. Bioengin.*, vol. 45, (1995), pp. 86-90.

Franěk, F. and Šramková, K. "Cell Suicide in Starving Hybridoma Culture: Survival-signal Effect of Some Amino Acids," *Cytotechnol.*, vol. 23, (1997), pp. 231-239.

Franěk, F. et al., "Plant Protein Hydrolysates: Preparation of Defined Peptide Fractions Promoting Growth and Production in Animal Cell Cultures," *Biotechnol. Prog.*, vol. 16, (2000), pp. 688-692.

Franěk, F. and Chládková-Šramková, K., "Apoptosis and Nutrition: Involvement of Amino Acid Transport System in Repression of Hybridoma Cell Death," *Cytotechnol.*, vol. 18, (1995), pp. 113-117.

Franěk, F. and Dolníková, J., "Hybridoma Growth and Monoclonal Antibody Production in Iron-rich Protein-free Medium: Effect of Nutrient Concentration," *Cytotechnol.*, vol. 7, (1991), pp. 33-38.

Jo, E-C. et al., "Balanced Nutrient Fortification Enables High-density Hybridoma Cell Culture in Batch Culture," *Biotechnol. Bioengin.*, vol. 36, (1990), pp. 717-720.

Franěk, F. and Šramková, K., "Protection of B Lymphocyte Hybridoma against Starvation-induced Apoptosis: Survival-signal role of Some Amino Acids," *Immunol. Lett.*, vol. 52, (1996), pp. 139-144.

Schneider, Y-J., "Optimization of Hybridoma Cell Growth and Monoclonal Antibody Secretion in a Chemically Defined, Serum- and Protein-free Culture Medium," *J. Immunol. Meth.*, vol. 116, (1989), pp. 65-77.

Keen, MJ., and Hale, C., "The Use of Serum-free Medium for the Production of Functionally Active Humanized Monoclonal Antibody from NS/0 Mouse Myeloma Cells Engineered Using Glutamine Synthetase as a Selectable Marker," *Cytotechnol.*, vol. 18, (1996) pp. 207-217.

Qi, YM. et al., "Evaluation of a Simple Protein-free Medium that Supports High Levels of Monoclonal Antibody Production," *Cytotechnol.*, vol. 21, (1996), pp. 95-109.

Franěk, F. and Šramková, K. "Cell Suicide in Starving Hybridoma Culture: Survival-signal Effect of Some Amino Acids," *Cytotechnol.*, vol. 21, (1996), pp. 81-89.

Cleveland, WL. et al., "Routine Large-scale Production of Monoclonal Antibodies in a Protein-free Culture Medium," *J. Immunol. Meth.*, vol. 56, (1983), pp. 221-234.

Chen, Z. et al., "A Low-cost Chemically Defined Protein-free Medium for a Recombinant CHO Cell Line Producing Prothrombin," *Biotechnol. Lett.*, vol. 22, (2000), pp. 837-841.

Tang, X-h. and Shay, NF. "Zinc Has an Insulin-like Effect on Glucose Transport Mediated by Phosphoinositol-3-kinase and Akt in 3T3-L1 Fibroblasts and Adipocytes," *J.Nutrit.*, vol. 131, (2001), pp. 1414-1420.

Ezaki, O. "IIb Group Metal Ions (Zn^{+2}, Cd^{+2}, and Hg^{+2}) Stimulate Glucose Transport Activity by Post-insulin Receptor Kinase Mechanism in Rat Adipocytes," *J.Biol.Chem.* vol. 264, (1989) pp. 16118-16122.

May, JM. And Contoreggi, CS., "The Mechanism of the Insulin-like Effects of Ionic Zinc," *J.Biol.Chem.*, vol. 257, (1982), pp. 4362-4368.

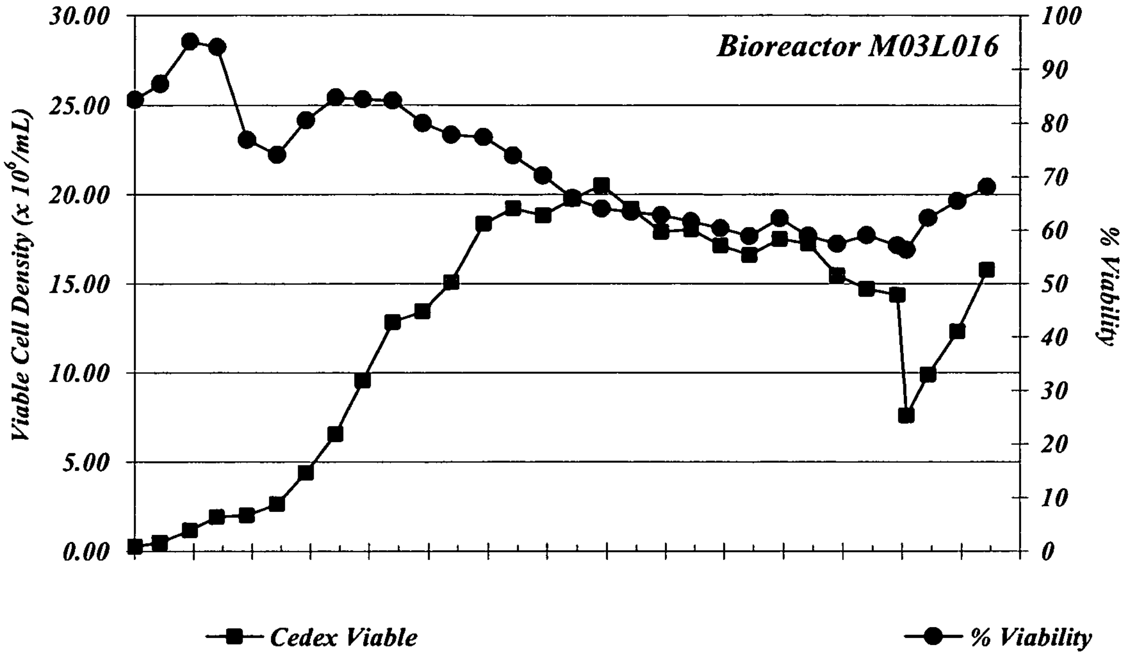
Primary Examiner—Lora E Barnhart
(74) *Attorney, Agent, or Firm*—Kirk Baumeister

(57) **ABSTRACT**

Chemically defined media compositions for the culture of eukaryotic cells are disclosed. The compositions are useful for eukaryotic cell culture in perfusion bioreactors and other vessels.

11 Claims, 3 Drawing Sheets

Fig. 1



Bioreactor M03L016
Viable Cell Density (x 10^6/mL)
% Viability
30.00
25.00
20.00
15.00
10.00
5.00
0.00
100
90
80
70
60
50
40
30
20
10
0
Cedex Viable
% Viability

Fig. 2

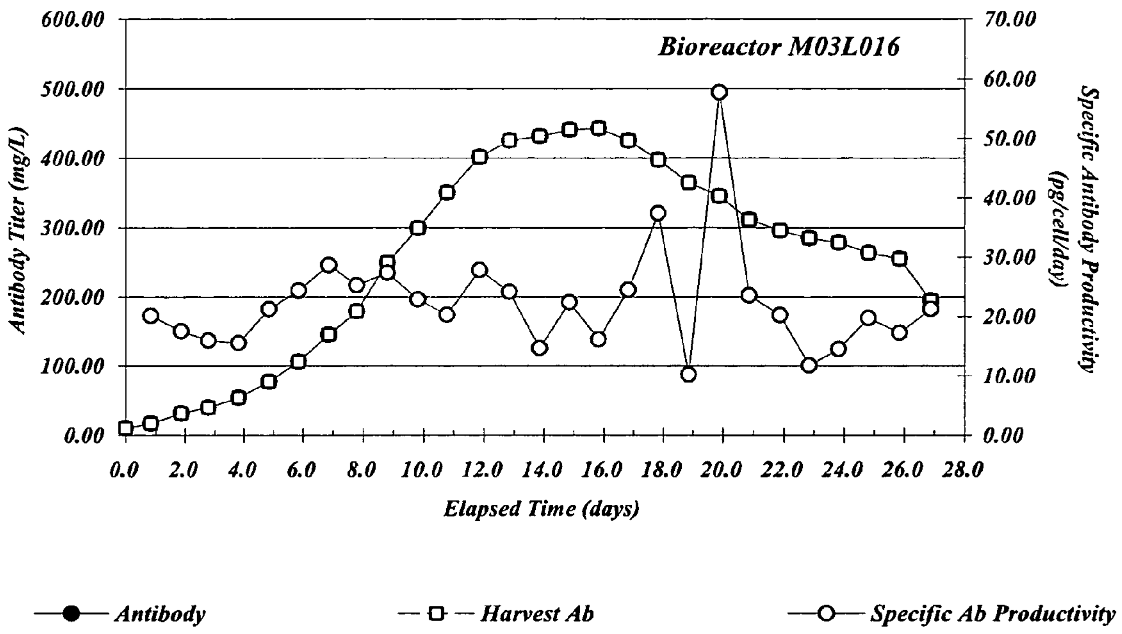
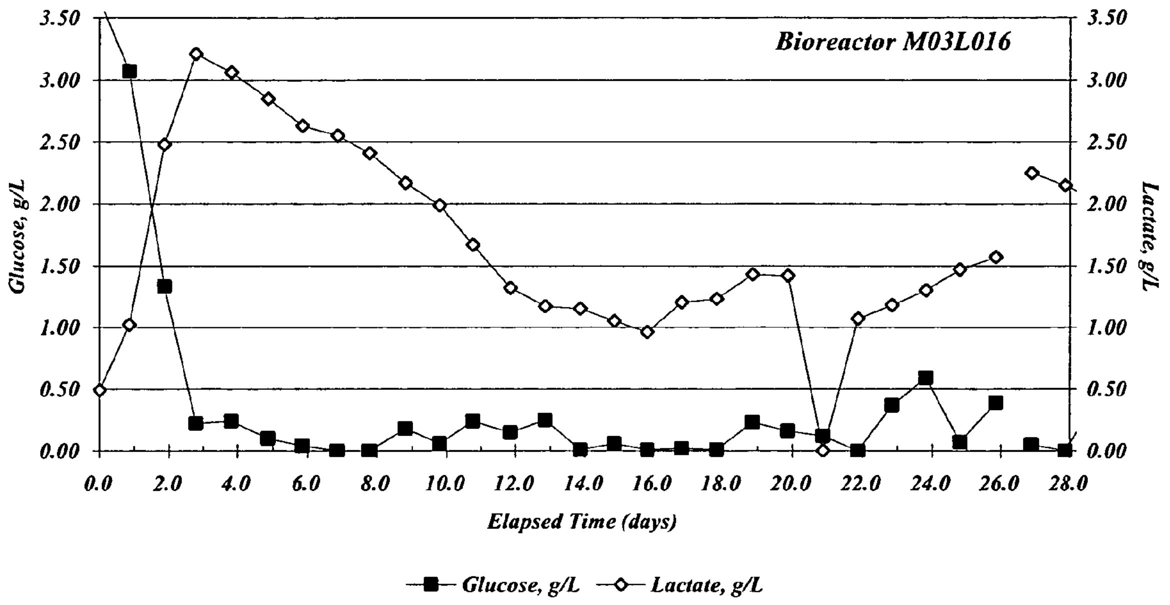

Bioreactor M03L016
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Antibody Titer (mg/L)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Specific Antibody Productivity (pg/cell/day)
0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0 22.0 24.0 26.0 28.0
Elapsed Time (days)
Antibody
Harvest Ab
Specific Ab Productivity

Fig. 3


Bioreactor M03L016
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Glucose, g/L
Lactate, g/L
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
22.0
24.0
26.0
28.0
Elapsed Time (days)
Glucose, g/L
Lactate, g/L

CHEMICALLY DEFINED MEDIA COMPOSITIONS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/623,718, filed 29 Oct. 2004, the entire contents of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to chemically defined media compositions for the culture of eukaryotic cells.

BACKGROUND OF THE INVENTION

Contamination of conventional eukaryotic cell culture media with "adventitious particles" such as bacterial, virus or prion particles is a serious potential problem in the industrial preparation of biopharmaceuticals such as antibodies or therapeutic proteins. Such contaminants in a biopharmaceutical are capable of causing patient infections and disease and may limit yields due to increased metabolic burdens on the host productioncell line.

Variant Creutzfeldt-Jakob disease (vCJD) is one example of a patient disease that could be caused by adventitious particle contamination. This disease is prion mediated in humans and is characterized by fatal neurodegeneration. vCJD has been strongly linked with exposure to the Bovine Spongiform Encephalopathy (BSE) prion which causes fatal, neurodegenerative "Mad Cow Disease" in cattle.

Adventitious particle contamination of conventional eukaryotic cell culture media can result from the incorporation of animal-derived components and protein growth factors into conventional media. Such contamination can occur when animal-derived media components are harvested from an animal harboring disease-causing bacteria, viruses, or prions. For example, bovine serum harvested from a cow with BSE may be contaminated with prions capable of causing human vCJD. The ultimate result of such adventitious particle contamination can be the contamination of eukaryotic cell cultures and the biopharmaceuticals prepared from such cultures.

Adventitious particle contamination can be avoided by culturing eukaryotic cells in animal component free cell culture media. Ideally, such media are "chemically defined" such that the media compositions contain only known chemical compounds, and are free of all proteins—even those not of animal origin such as recombinant proteins.

Chemically defined media compositions optimal for production of biopharmaceuticals, such as antibodies, must satisfy several different criteria. First, such compositions must limit eukaryotic cell damage resulting from shear forces and other cell-damaging processes that occur in the bioreactor vessels typically used for biopharmaceutical production. Second, such compositions must enable eukaryotic cell cultures to have high viable cell densities (i.e., number viable cells/ml media) and high percentages of viable cells. Third, such compositions must permit high titers of secreted biopharmaceutical products (i.e., antibody mg/L media) and high specific productivities (i.e., pg antibody/viable cell/day). Lastly, such compositions must limit the production of lactic acid by cultured eukaryotic cells to permit the most efficient cellular use of glucose.

Thus, a need exists for chemically defined media compositions which satisfy these criteria and are optimized for biopharmaceutical production.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Eukaryotic cell viability in MET 1.5 cell culture media.

FIG. **2**. Antibody titer and specific productivity in MET 1.5 cell culture media.

FIG. **3**. Decreased lactate production in MET 1.5 cell culture media.

SUMMARY OF THE INVENTION

One aspect of the invention is a soluble composition, suitable for producing a cell culture media, wherein the media comprises the following components in the following amounts per liter:

anhydrous $CaCl_2$, 5-200 mg;
anhydrous $MgCl_2$, 15-50 mg;
anhydrous $MgSO_4$, 20-80 mg;
$FeSO_4.7H_2O$, 0.05-0.50 mg;
$Fe(NO_3)_3.9H_2O$, 0.01-0.08 mg;
$ZnSO_4.7H_2O$, 0.40-1.20 mg;
ferric ammonium citrate, 0.04-200 mg;
KCl, 280-500 mg;
NaCl, 5000-7500 mg;
NaH_2PO_4—H_2O, 30-100 mg;
Na_2HPO_4, 30-100 mg;
$CuSO_4.5H_2O$, 0.001-0.005 mg;
$CoCl_2.6H_2O$, 0.001-0.10 mg;
$(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.001-0.005 mg;
$MnSO_4$—H_2O, 0.000070-0.0080 mg;
$NiSO_4.6H_2O$, 0.000025-0.0005 mg;
Na_2SeO_3, 0.004-0.07 mg;
$Na_2SiO_3.9H_2O$, 0.02-0.4 mg;
$SnCl_2.2H_2O$, 0.000025-0.0005 mg;
NH_4VO_3, 0.0001-0.0025 mg;
D-Glucose, 500-8000 mg;
sodium pyruvate, 0.0-1000 mg;
sodium hypoxanthine, 0.0-20.0 mg;
glycine, 0.0-150 mg;
L-alanine, 0.0-150 mg;
L-arginine.HCl, 200-5000 mg;
L-asparagine.H_2O, 40-250 mg;
L-aspartic acid, 20-1000 mg;
L-cysteine.HCl H_2O, 25.0-250 mg;
L-cystine.2HCl, 15-150 mg;
L-glutamic acid, 0-1000 mg;
L-histidine.HCl.H_2O, 100-500 mg;
L-isoleucine, 50-1000 mg;
L-leucine, 50-1000 mg;
L-lysine.HCl, 100-1000 mg;
L-methionine, 50-500 mg;
L-ornithine.HCl, 0-100 mg;
L-phenylalanine, 25-1000 mg;
L-proline, 0-1000 mg;
L-serine, 50-500 mg;
L-taurine, 0-1000 mg;
L-threonine, 50-600 mg;
L-tryptophan, 2-500 mg;
L-tyrosine.2Na.$2H_2O$, 25-250 mg;
L-valine, 100-1000 mg;
d-biotin, 0.04-1.0 mg;
D-calcium pantothenate, 0.1-5.0 mg;
choline chloride, 1-100 mg;
folic acid, 1-10 mg;
i-Inositol, 10-1000 mg;
nicotinamide, 0.5-30 mg;
p-aminobenzoic acid, 0.1-20 mg;

riboflavin, 0.05-5.0 mg;
thiamine.HCl, 0.5-20 mg;
thymidine, 0-3.0 mg;
vitamin B_{12}, 0.05-5.0 mg;
linoleic acid, 0.01-2.0 mg;
DL-α-lipoic acid, 0.03-1.0 mg;
pyridoxine.HCl, 0.5-30 mg;
putrescine.2HCl, 0.025-0.25 mg; and
ethanolamine.HCl, 2-100 mg.

Another aspect of the invention is a soluble composition, suitable for producing a cell culture media, wherein the media comprises the following components in the following amounts per liter:

$CaCl_2$, 100.95 mg;
$MgCl_2$, 24.77 mg;
$MgSO_4$, 42.24 mg;
$FeSO_4.7H_2O$, 0.3607 mg;
$Fe(NO_3)_3.9H_2O$, 0.0432 mg;
$ZnSO_4.7H_2O$, 0.6225 mg;
ferric ammonium citrate, 43.25 mg;
KCl, 386.9 mg;
NaCl, 5866.0 mg;
$NaH_2PO_4—H_2O$, 54.07 mg;
Na_2HPO_4, 61.44 mg;
$CuSO_4.5H_2O$, 0.003287 mg;
$CoCl_2.6H_2O$, 0.0020606 mg;
$(NH_4)_6Mo_7O_{24}.4H_2O$, 0.000535 mg;
$MnSO_4.H_2O$, 0.00008571 mg;
$NiSO_4.6H_2O$, 0.0000514 mg;
Na_2SeO_3, 0.007489 mg;
$Na_2SiO_3.9H_2O$, 0.03671 mg;
$SnCl_2.2H_2O$, 0.0000488 mg;
NH_4VO_3, 0.0002530 mg;
D-Glucose, 3680.52 mg;
sodium pyruvate, 100 mg;
sodium hypoxanthine, 2.069 mg;
glycine, 16.23 mg;
L-alanine, 79.31 mg;
L-arginine.HCl, 674.89 mg;
L-asparagine.H_2O, 182.25 mg;
L-aspartic acid, 67.23 mg;
L-cysteine.HCl.H_2O, 57.63 mg;
L-cystine.2HCl, 106.70 mg;
L-glutamic acid, 6.36 mg;
L-histidine.HCl.H_2O, 250.55 mg;
L-isoleucine, 245.43 mg;
L-leucine, 263.42 mg;
L-lysine.HCl, 276.41 mg;
L-methionine, 85.40 mg;
L-ornithine.HCl, 2.44 mg;
L-phenylalanine, 104.23 mg;
L-proline, 14.94 mg;
L-serine, 146.36 mg;
L-taurine, 3.64 mg;
L-threonine, 199.09 mg;
L-tryptophan, 70.71 mg;
L-tyrosine.2Na.2H_2O, 195.58 mg;
L-valine, 174.34 mg;
d-biotin, 0.4359 mg;
D-calcium pantothenate, 1.9394 mg;
choline chloride, 10.8009 mg;
folic acid, 3.4329 mg;
i-inositol, 81.7965 mg;
nicotinamide, 3.1342 mg;
p-aminobenzoic acid, 2.1645 mg;
riboflavin, 0.5359 mg;
thiamine.HCl, 2.3377 mg;
thymidine, 0.316 mg;
vitamin B_{12}, 0.5887 mg;
linoleic acid, 0.0364 mg;
DL-α-lipoic acid, 0.0909 mg;
pyridoxine.HCl, 3.0442 mg;
putrescine.2HCl, 0.0701 mg; and
ethanolamine.HCl, 14.37 mg.

The invention also provides compositions comprising cell culture media which can be made from the soluble compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

The term "buffering molecule" as used herein and in the claims means a molecule that has a buffering range suitable for maintaining a pH between 5.9 and 7.8.

The term "pK_a" as used herein and in the claims means the negative logarithm of the acid dissociation constant (K_a) of a buffering molecule in an aqueous solution. pK_a is, in part, a function of the temperature of the aqueous solution in which a buffering molecule is solubilized.

The term "cell protectant" as used herein and in the claims means a substance that protects eukaryotic cells from damage. Such damage may be caused, for example, by shear forces or the effects of gas bubble sparging in a bioreactor vessel.

The present invention provides chemically defined compositions useful in the culture of eukaryotic cells. Such eukaryotic cells may have insect, avian, mammalian, or other origins. These cells may secrete a protein, such as an antibody, or produce other useful products or results. These proteins, products, or results may be constituatively produced by a cell or produced as the result of transfection with a nucleic acid sequence. The cells may be cultured in liquid media as suspension cultures or as adherent cultures. Cells may also be cultured by suspension in semi-solid media comprising the compositions of the invention.

Cells may be cultured in a variety of vessels including, for example, perfusion bioreactors, cell bags, culture plates, flasks and other vessels well known to those of ordinary skill in the art. Ambient conditions suitable for cell culture, such as temperature and atmospheric composition, are also well known to those skilled in the art. Methods for the culture of cells are also well known to those skilled in the art.

The compositions of the invention are particularly useful in the culture of mammalian cells. Examples of mammalian cells include myeloma derived cells, non-immortalized cells of the B cell lineage, and immortalized cells of the B cell lineage such as hybridomas. Examples of myeloma derived cell lines include the SP2/0 (American Type Culture Collection (ATCC), Manasas, Va., CRL-1581), NSO (European Collection of Cell Cultures (ECACC), Salisbury, Wiltshire, UK, ECACC No. 85110503), FO (ATCC CRL-1646), and Ag653 (ATCC CRL-1580) cell lines which were obtained from mice. The C743B cell line is an example of a SP2/0 derived cell line that produces a fully human, anti-IL-12 mAb as the result of stable transfection. The YB2/0 cell line (ATCC CRL-1662) is an example of a myeloma derived cell line obtained from rats (*Rattus norvegicus*). An example of a myeloma derived cell line obtained from humans is the U266 cell line (ATTC CRL-TIB-196). Some myeloma derived cell lines, such as NSO, YB2/0, and Ag653 cells and related cell lines may require chemically defined lipid concentrates or other supplements for successful culture. Those skilled in the art will recognize other myeloma cell lines and myeloma derived cell lines as well as any supplements required for the successful culture of such cells.

In one aspect the invention provides a soluble composition, suitable for producing a cell culture media, wherein the media comprises the following components in the following amounts per liter:

anhydrous $CaCl_2$, 5-200 mg;
anhydrous $MgCl_2$, 15-50 mg;
anhydrous $MgSO_4$, 20-80 mg;
$FeSO_4.7H_2O$, 0.05-0.50 mg;
$Fe(NO_3)_3.9H_2O$, 0.01-0.08 mg;
$ZnSO_4.7H_2O$, 0.40-1.20 mg;
ferric ammonium citrate, 0.04-200 mg;
KCl, 280-500 mg;
NaCl, 5000-7500 mg;
$NaH_2PO_4.H_2O$, 30-100 mg;
Na_2HPO_4, 30-100 mg;
$CuSO_4.5H_2O$, 0.001-0.005 mg;
$CoCl_2.6H_2O$, 0.001-0.10 mg;
$(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.001-0.005 mg;
$MnSO_4.H_2O$, 0.000070-0.0080 mg;
$NiSO_4.6H_2O$, 0.000025-0.0005 mg;
Na_2SeO_3, 0.004-0.07 mg;
$Na_2SiO_3.9H_2O$, 0.02-0.4 mg;
$SnCl_2.2H_2O$, 0.000025-0.0005 mg;
NH_4VO_3, 0.0001-0.0025 mg;
D-Glucose, 500-8000 mg;
sodium pyruvate, 0.0-1000 mg;
sodium hypoxanthine, 0.0-20.0 mg;
glycine, 0.0-150 mg;
L-alanine, 0.0-150 mg;
L-arginine.HCl, 200-5000 mg;
L-asparagine.H_2O, 40-250 mg;
L-aspartic acid, 20-1000 mg;
L-cysteine.HCl H_2O, 25.0-250 mg;
L-cystine.2HCl, 15-150 mg;
L-glutamic acid, 0-1000 mg;
L-histidine.HCl.H_2O, 100-500 mg;
L-isoleucine, 50-1000 mg;
L-leucine, 50-1000 mg;
L-lysine.HCl, 100-1000 mg;
L-methionine, 50-500 mg;
L-ornithine.HCl, 0-100 mg;
L-phenylalanine, 25-1000 mg;
L-proline, 0-1000 mg;
L-serine, 50-500 mg;
L-taurine, 0-1000 mg;
L-threonine, 50-600 mg;
L-tryptophan, 2-500 mg;
L-tyrosine-2Na-$2H_2O$, 25-250 mg;
L-valine, 100-1000 mg;
d-biotin, 0.04-1.0 mg;
D-calcium pantothenate, 0.1-5.0 mg; choline chloride, 1-100 mg;
folic acid, 1-10 mg;
i-Inositol, 10-1000 mg;
nicotinamide, 0.5-30 mg;
p-aminobenzoic acid, 0.1-20 mg;
riboflavin, 0.05-5.0 mg;
thiamine.HCl, 0.5-20 mg;
thymidine, 0-3.0 mg;
vitamin B_{12}, 0.05-5.0 mg;
linoleic acid, 0.01-2.0 mg;
DL-α-lipoic acid, 0.03-1.0 mg;
pyridoxine.HCl, 0.5-30 mg;
putrescine.2HCl, 0.025-0.25 mg; and
ethanolamine.HCl, 2-100 mg.

This type of soluble composition has been named "MET" and typically is a powder.

In another aspect the invention provides a soluble composition, suitable for producing a cell culture media, wherein the media comprises the following components in the following amounts per liter:

$CaCl_2$, 100.95 mg;
$MgCl_2$, 24.77 mg;
$MgSO_4$, 42.24 mg;
$FeSO_4.7H_2O$, 0.3607 mg;
$Fe(NO_3)_3.9H_2O$, 0.0432 mg;
$ZnSO_4.7H_2O$, 0.6225 mg;
ferric ammonium citrate, 43.25 mg;
KCl, 386.9 mg;
NaCl, 5866.0 mg;
$NaH_2PO_4.H_2O$, 54.07 mg;
Na_2HPO_4, 61.44 mg;
$CuSO_4.5H_2O$, 0.003287 mg;
$CoCl_2.6H_2O$, 0.0020606 mg;
$(NH_4)_6Mo_7O_{24}.4H_2O$, 0.000535 mg;
$MnSO_4.H_2O$, 0.00008571 mg;
$NiSO_4.6H_2O$, 0.0000514 mg;
Na_2SeO_3, 0.007489 mg;
$Na_2SiO_3.9H_2O$, 0.03671 mg;
$SnCl_2.2H_2O$, 0.0000488 mg;
NH_4VO_3, 0.0002530 mg;
D-Glucose, 3680.52 mg;
sodium pyruvate, 100 mg;
sodium hypoxanthine, 2.069 mg;
glycine, 16.23 mg;
L-alanine, 79.31 mg;
L-arginine.HCl, 674.89 mg;
L-asparagine.H_2O, 182.25 mg;
L-aspartic acid, 67.23 mg;
L-cysteine.HCl.H_2O, 57.63 mg;
L-cystine.2HCl, 106.70 mg;
L-glutamic acid, 6.36 mg;
L-histidine.HCl.H_2O, 250.55 mg;
L-isoleucine, 245.43 mg;
L-leucine, 263.42 mg;
L-lysine-HCl, 276.41 mg;
L-methionine, 85.40 mg;
L-ornithine-HCl, 2.44 mg;
L-phenylalanine, 104.23 mg;
L-proline, 14.94 mg;
L-serine, 146.36 mg;
L-taurine, 3.64 mg;
L-threonine, 199.09 mg;
L-tryptophan, 70.71 mg;
L-tyrosine.2Na.$2H_2O$, 195.58 mg;
L-valine, 174.34 mg;
d-biotin, 0.4359 mg;
D-calcium pantothenate, 1.9394 mg;
choline chloride, 10.8009 mg;
folic acid, 3.4329 mg;
i-inositol, 81.7965 mg;
nicotinamide, 3.1342 mg;
p-aminobenzoic acid, 2.1645 mg;
riboflavin, 0.5359 mg;
thiamine.HCl, 2.3377 mg;
thymidine, 0.316 mg;
vitamin B_{12}, 0.5887 mg;
linoleic acid, 0.0364 mg;
DL-α-lipoic acid, 0.0909 mg;

pyridoxine.HCl, 3.0442 mg;
putrescine.2HCl, 0.0701 mg; and
ethanolamine.HCl, 14.37 mg.

This soluble composition has been named "MET 1.5" and typically is a powder.

In one embodiment the soluble MET and MET 1.5 compositions of the invention comprise a buffering molecule with a pK_a of between 5.9 and 7.8; and a cell protectant. Examples of buffering molecules with a pK_a of between 5.9 and 7.8 include MOPS (pK_a 7.20 at 25° C.; pK_a 7.02 at 37° C.), TES (2-[tris (hydroxymothyl) methyl]amino ethanesulphonic acid; pK_a 7.40 at 25° C.; pK_a 7.16 at 37° C.), and imidazole (pK_a 6.95 at 25° C.). Examples of cell protectants are non-ionic surfactants such as Pluronic-F68, polyvinyl alcohol (PVA), polyethylene glycol (PEG), and dextran sulfate. Those skilled in the art will recognize other buffering molecules with a pK_a of between 5.9 and 7.8 and cell protectants.

In another embodiment of the soluble MET compositions of the invention the buffering molecule consists of MOPS in the amount of 1047-5230 mg per liter of media volume, and the cell protectant consists of Pluronic-F68 in the amount of 250-1500 mg per liter of media volume.

In another embodiment of the soluble MET1.5 compositions of the invention the buffering molecule consists of MOPS in the amount of 2709.66 mg per liter of media volume, and the cell protectant consists of Pluronic-F68 in the amount of 865.80 mg per liter of media volume.

The soluble compositions of the invention may be prepared in a variety of forms. It is preferred that the soluble compositions of the invention are prepared in the form of a powder. The powdered forms of the soluble compositions of the invention are suitable for cell culture for at least 3 years from the date the soluble composition is prepared. The soluble compositions of the invention may also be prepared, for example, in the form of one or more pellets or tablets.

The soluble compositions of the invention can be solubilized in water. Typically, the water used to solubilize the soluble compositions of the invention has a resistivity of 18.2 MΩ·cm at 25° C., a total organic content of less than 20 ppb, a total microorganism content of less than 10 colony forming units per ml, a total heavy metal content of less than 0.01 ppm, a total silicates content of less than 0.01 ppb, and a total dissolved solids content of less than 0.03 ppm. Water with these properties can be prepared using a Super-Q™ Plus Water Purification System (Millipore Corp., Billerica, Mass., USA). The water used to solubilize the soluble compositions of the invention may also be filtered through a filter suitable for the removal of microorganisms. A filter with a 0.22 μM pore size is an example of such a filter. Microorganisms and other adventitious particles may also be removed or inactivated by other means well known in the art.

In one embodiment the invention provides a composition comprising a cell culture media made by the steps comprising selecting a final media volume, providing a soluble MET composition, solubilizing the soluble composition in a volume of water less than the final media volume, adding 1.022 g of L-glutamine per liter of final media volume, adding a bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume, optionally adding at least one substance selected from the group consisting of mycophenolic acid, hypoxanthine, xanthine, or soy hydrosylate, adding a quantity of base sufficient to adjust the pH of the solution to between pH 5.9 and pH 7.8, and adding water sufficient to bring the volume of the composition to the selected final media volume. In this embodiment of the invention the media composition that is the product of this process has been named "MET media." Typically MET media is a liquid media.

In another embodiment the invention provides a composition comprising a cell culture media made by the steps comprising selecting a final media volume; providing a soluble MET1.5 composition, solubilizing the soluble composition in a volume of water less than the final media volume, adding 1.022 g of L-glutamine per liter of final media volume, adding a bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume, optionally adding at least one substance selected from the group consisting of mycophenolic acid, hypoxanthine, xanthine or soy hydrosylate, adding a quantity of base sufficient to adjust the pH of the solution to between pH 5.9 and pH 7.8, and adding water sufficient to bring the volume of the composition to the selected final media volume. In this embodiment of the invention the media composition that is the product of this process has been named "MET 1.5 media." Typically MET 1.5 media is a liquid media.

In one embodiment of the invention the bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume is 2.1 g of $NaHCO_3$ per liter of final media volume. Adding this amount of $NaHCO_3$ per liter of final media volume produces a bicarbonate ion concentration of 0.025 M in the final media volume.

In one embodiment of the invention MET 1.5 media comprises the following components added in the following amounts per liter:

0.5 mg mycophenolic acid;
2.5 mg hypoxanthine; and
50 mg xanthine.

The MET media and MET 1.5 media compositions of the invention are typically provided to cells as a liquid media. The pH of the MET media and MET 1.5 media compositions of the invention is between pH 5.9 and pH 7.8. The pH of a liquid is a function of the temperature of the liquid. It is preferred that the pH of each media composition be between 7.1 and 7.25 at the temperature at which eukaryotic cell culture is being performed. Eukaryotic cell culture may be performed at temperatures higher or lower than 37° C., but is typically performed at 37° C.

In some applications liquid MET media and liquid MET 1.5 media may be used in the preparation of semi-solid cell culture media. For example, methylcellulose may be used to generate a semi-solid media incorporating the liquid MET media and liquid MET 1.5 media compositions of the invention. Such semi-solid media may be prepared by methods well known to those skilled in the art. Eukaryotic cells may be suspended in such semi-solid media and cultured by methods well known to those skilled in the art.

Other substances that can enhance cell growth or productivity may also be added to the soluble MET, MET media, soluble MET 1.5 and MET 1.5 media compositions of the invention. These substances may be lipids, nucleosides, peptide chains, corticosteroids, steroids, and the like. Such substance may be, for example:

adenosine preferably 0-20 μM;
guanosine preferably 0-20 μM;
cytidine preferably 0-20 μM;
uridine preferably 0-20 μM;
deoxyadenosine preferably 0-20 μM;
deoxyguanosine preferably 0-20 μM;
deoxycytidine preferably 0-20 μM;
thymidine preferably 0-20 μM;

dexamethasone preferably 10-150 nM;
hydrocortisone preferably 0-150 μM;
L-glycine-L-Lysine-L-glycine (GKG) peptide chain preferably 0-200 μm;
N-acetyl cysteine preferably 0-500 mg/L;
betaine preferably 0-500 mg/L;
L-malic acid preferably 0-500 mg/L;
oxaloacetic acid preferably 0-500 mg/L;
glycyrrhizic acid preferably 0-500 mg/L;
glycyrrhizic acid ammonium salt preferably 0-500 mg/L;
α-ketoglutarate preferably 0-500 mg/L;
L-leucine preferably 245-490 mg/L;
L-isoleucine preferably 220-440 mg/L;
L-lysine-HCl preferably 187-360 mg/L;
L-valine preferably 155-310 mg/L;
L-methionine preferably 57-114 mg/L;
L-phenylalanine preferably 76-152 mg/L;
L-serine preferably 37-74 mg/L;
L-threonine preferably 107-214 mg/L;
L-arginine.HCl preferably 200-300 mg/L;
L-asparagine preferably 114-170 mg/L;
L-aspartic acid (10-25 mg/L);
L-cysteine.HCl.H_2O preferably 46-75 mg/L;
Histidine.HCl.H_2O preferably 75-150 mg/L;
L-tyrosine preferably 40-80 mg/L;
L-tryptophan preferably 41-82 mg/L;
nicotinamide preferably 0.9-1.8 mg/L; and
ethanolamine HCl preferably 14-20 mg/L.

The quantities of each substance added to the compositions of the invention are those necessary to achieve the preferred molar concentration or mass per unit media volume prepared shown above.

The present invention is further described with reference to the following examples. These examples are merely to illustrate various aspects of the present invention and are not intended as limitations of this invention.

EXAMPLE 1

Eukaryotic Cell Viability in MET 1.5 Cell Culture Media

Chemically defined MET 1.5 cell culture media can sustain high cell growth and viability (FIG. **1**). To examine viable cell numbers, MET 1.5 media was supplied to 3 L perfusion bioreactors. Bioreactors were then inoculated with C743B cells such that the initial cell density was 3×10^6 cells/ml of MET 1.5 media. The C743B cell line produces a fully human, anti-IL-12 mAb and is a chemically adapted cell line derived from SP2/0 myeloma cells. C743B cells were grown for 29 days in the bioreactor and viable cell densities were monitored. Cell culture media was neutralized with a 0.2 M Na_2CO_3 (aq) solution for the first 9 days of culture and with 0.2 M Na_2CO_3, 0.0054 M K_2CO_3 (aq). Excessive cell density in the bioreactor was prevented by the removed of biomass from the bioreactor; cell removal began on day 15 and was gradually increased until day 26. The bioreactor was perfused with one volume of MET 1.5 media per day. Viable cell numbers were determined via a standard trypan blue dye exclusion assay using a CEDEX cell counter (Innovatis AG, Bielefeld, Del.). Total cell numbers for calculation of the percentage of viable cells were determined with the CEDEX instrument. For each determination the CEDEX instrument was used according to the manufacturer's instructions. O_2 and CO_2 were supplied to the bioreactor as a gas stream sparged into the bioreactor vessel. Data presented in Example 1, 2, and 3 are all from the same bioreactor run.

EXAMPLE 2

Antibody Titer and Specific Productivity in MET 1.5 Cell Culture Media

Chemically defined MET 1.5 cell culture media can sustain high monoclonal antibody titers and specific productivity (FIG. **2**). Cell culture and bioreactor operation was as described above. Fully human, anti-IL-12 mAb titers (mg/L) were determined by standard nephelometry techniques using a Beckman Array Analyzer. A purified fully human, anti-IL-12 mAb of known concentration was used to generate a standard curve for the determination of mAb titers by nephelometry. Viable cell numbers for calculation of specific productivity were determined as described above. Data presented in Example 1, 2, and 3 are all from the same bioreactor run.

EXAMPLE 3

Decreased Lactate Production in MET 1.5 Cell Culture Media

Lactate concentrations in MET 1.5 media decrease (FIG. **3**) as viable cell density increases (FIG. **1**). Cell culture and bioreactor operation was as described above. Lactate concentrations and glucose concentrations in the bioreactor culture media were determined using standard assays. Data presented in Example 1, 2, and 3 are all from the same bioreactor run.

As FIG. **3** indicates, lactate concentrations in MET 1.5 media gradually decreased until day 16 when biomass removal to decrease total cell density in the bioreactor began. During the same period glucose concentrations remained comparatively constant (FIG. **3**). Comparison of FIG. **3** to FIG. **1** reveals that viable C743B cell numbers in the same bioreactor were increasing until day 16. Together this data indicates a decrease in lactate production by C743B cells cultured in MET 1.5 media and more efficient metabolism of D-glucose by cells cultured in MET 1.5 media.

The present invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

The invention claimed is:

1. A soluble composition, suitable for producing a final volume of cell culture media, wherein the composition comprises the following components in the following amounts per liter of the final volume of cell culture media:

anhydrous $CaCl_2$, 5-200 mg;
anhydrous $MgCl_2$, 15-50 mg;
anhydrous $MgSO_4$, 20-80 mg;
$FeSO_4.7H_2O$, 0.05-0.50 mg;
$Fe(NO_3)_3.9H_2O$, 0.01-0.08 mg;
$ZnSO_4.7H_2O$, 0.40-1.20 mg;
ferric ammonium citrate, 0.04-200 mg;
KCl, 280-500 mg;
NaCl, 5000-7500 mg;
$NaH_2PO_4.H_2O$, 30-100 mg;
Na_2HPO_4, 30-100 mg;
$CuSO_4.5H_2O$, 0.001-0.005 mg;
$CoCl_2.6H_2O$, 0.001-0.10 mg;
$(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.001-0.005 mg;
$MnSO_4.H_2O$, 0.000070-0.0080 mg;

$NiSO_4.6H_2O$, 0.000025-0.0005 mg;
Na_2SeO_3, 0.004-0.07 mg;
$Na_2SiO_3.9H_2O$, 0.02-0.4 mg;
$SnCl_2.2H_2O$, 0.000025-0.0005 mg;
NH_4VO_3, 0.0001-0.0025 mg;
D-Glucose, 500-8000 mg;
sodium pyruvate, 0.0-1000 mg;
sodium hypoxanthine, 0.0-20.0 mg;
glycine, 0.0-150 mg;
L-alanine, 0.0-150 mg;
L-arginine.HCl, 200-5000 mg;
L-asparagine.H_2O, 40-250 mg;
L-aspartic acid, 20-1000 mg;
L-cysteine.HCl H_2O, 25.0-250 mg;
L-cystine.2HCl, 15-150 mg;
L-glutamic acid, 0-1000 mg;
L-histidine.HCl.H_2O, 100-500 mg;
L-isoleucine, 50-1000 mg;
L-leucine, 50-1000 mg;
L-lysine.HCl, 100-1000 mg;
L-methionine, 50-500 mg;
L-ornithine.HCl, 0-100 mg;
L-phenylalanine, 25-1000 mg;
L-proline, 0-1000 mg;
L-serine, 50-500 mg;
L-taurine, 0-1000 mg;
L-threonine, 50-600 mg;
L-tryptophan, 2-500 mg;
L-tyrosine.2Na.2H_2O, 25-250 mg;
L-valine, 100-1000 mg;
d-biotin, 0.04-1.0 mg;
D-calcium pantothenate, 0.1-5.0 mg;
choline chloride, 1-100 mg;
folic acid, 1-10 mg;
i-Inositol, 10-1000 mg;
nicotinamide, 0.5-30 mg;
p-aminobenzoic acid, 0.1-20 mg;
riboflavin, 0.05-5.0 mg;
thiamine.HCl, 0.5-20 mg;
thymidine, 0-3.0 mg;
vitamin B_{12}, 0.05-5.0 mg;
linoleic acid, 0.01-2.0 mg;
DL-α-lipoic acid, 0.03-1.0 mg;
pyridoxine.HCl, 0.5-30 mg;
putrescine.2HCl, 0.025-0.25 mg; and
ethanolamine.HCl, 2-100 mg.

2. The soluble composition of claim **1** further comprising a buffering molecule with a pK_a between 5.9 and 7.8 and a cell protectant.

3. The soluble composition of claim **2** wherein the buffering molecule consists of MOPS in the amount of 1047-5230 mg per liter of final media volume and the cell protectant consists of Pluronic-F68 in the amount of 250-1500 mg per liter of final media volume.

4. A composition comprising a cell culture media made by the steps comprising:

a) selecting a final media volume;
b) providing the soluble composition of claim **2** or claim **3**;
c) solubilizing the soluble composition in a volume of water less than the final media volume;
d) adding 1.022 g of L-glutamine per liter of final media volume;
e) adding a bicarbonate ion providing substance sufficient to produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume;
f) optionally adding at least one substance selected from the group consisting of mycophenolic acid, hypoxanthine, xanthine, and soy hydrolysate;
g) adding a quantity of base sufficient to adjust the pH of the solution to between pH 5.9 and pH 7.8; and
h) adding water sufficient to bring the volume of the composition to the selected final media volume.

5. The composition of claim **4** where the bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume is 2.1 g of $NaHCO_3$ per liter of final media volume.

6. A soluble composition, suitable for producing a final volume of cell culture media, wherein the composition comprises the following components in the following amounts per liter of the final volume of cell culture media:

$CaCl_2$, 100.95 mg;
$MgCl_2$, 24.77 mg;
$MgSO_4$, 42.24 mg;
$FeSO_4.7H_2O$, 0.3607 mg;
$Fe(NO_3)_3.9H_2O$, 0.0432 mg;
$ZnSO_4.7H_2O$, 0.6225 mg;
ferric ammonium citrate, 43.25 mg;
KCl, 386.9 mg;
NaCl, 5866.0 mg;
$NaH_2PO_4.H_2O$, 54.07 mg;
Na_2HPO_4, 61.44 mg;
$CuSO_4.5H_2O$, 0.003287 mg;
$CoCl_2.6H_2O$, 0.0020606 mg;
$(NH_4)_6Mo_7O_{24}.4H_2O$, 0.000535 mg;
$MnSO_4.H_2O$, 0.00008571 mg;
$NiSO_4.6H_2O$, 0.0000514 mg;
Na_2SeO_3, 0.007489 mg;
$Na_2SiO_3.9H_2O$, 0.03671 mg;
$SnCl_2.2H_2O$, 0.0000488 mg;
NH_4VO_3, 0.0002530 mg;
D-Glucose, 3680.52 mg;
sodium pyruvate, 100 mg;
sodium hypoxanthine, 2.069 mg;
glycine, 16.23 mg;
L-alanine, 79.31 mg;
L-arginine.HCl, 674.89 mg;
L-asparagine.H_2O, 182.25 mg;
L-aspartic acid, 67.23 mg;
L-cysteine.HCl.H_2O, 57.63 mg;
L-cystine.2HCl, 106.70 mg;
L-glutamic acid, 6.36 mg;
L-histidine.HCl.H_2O, 250.55 mg;
L-isoleucine, 245.43 mg;
L-leucine, 263.42 mg;
L-lysine.HCl, 276.41 mg;
L-methionine, 85.40 mg;
L-ornithine.HCl, 2.44 mg;
L-phenylalanine, 104.23 mg;
L-proline, 14.94 mg;
L-serine, 146.36 mg;
L-taurine, 3.64 mg;
L-threonine, 199.09 mg;
L-tryptophan, 70.71 mg;
L-tyrosine.2Na.2H_2O, 195.58 mg;
L-valine, 174.34 mg;
d-biotin, 0.4359 mg;
D-calcium pantothenate, 1.9394 mg;
choline chloride, 10.8009 mg;
folic acid, 3.4329 mg;
i-inositol, 81.7965 mg;
nicotinamide, 3.1342 mg;

p-aminobenzoic acid, 2.1645 mg;
riboflavin, 0.5359 mg;
thiamine.HCl, 2.3377 mg;
thymidine, 0.316 mg;
vitamin B_{12}, 0.5887 mg;
linoleic acid, 0.0364 mg;
DL-α-lipoic acid, 0.0909 mg;
pyridoxine.HCl, 3.0442 mg;
putrescine.2HCl, 0.0701 mg; and
ethanolamine.HCl, 14.37 mg.

7. The soluble composition of claim **6** further comprising a buffering molecule with a pK_a of between 5.9 and 7.8 and a cell protectant.

8. The soluble composition of claim **7** wherein the buffering molecule consists of MOPS in the amount of 2709.66 mg per liter of final media volume, and the cell protectant consists of Pluronic-F68 in the amount of 865.80 mg per liter of final media volume.

9. The soluble composition of claim **7** further comprising the following components in the following amounts per liter of final media volume:

0.5 mg mycophenolic acid;
2.5 mg hypoxanthine; and
50 mg xanthine.

10. A composition comprising a cell culture media made by the steps comprising:

a) selecting a final media volume;
b) providing the soluble composition of claim **7** or claim **8**;
c) solubilizing the soluble composition in a volume of water less than the final media volume;
d) adding 1.022 g of L-glutamine per liter of final media volume;
e) adding a bicarbonate ion providing substance sufficient to produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume;
f) optionally adding at least one substance selected from the group consisting of mycophenolic acid, hypoxanthine, xanthine and soy hydrolysate;
g) adding a quantity of base sufficient to adjust the pH of the solution to between pH 5.9 and pH 7.8; and
h) adding water sufficient to bring the volume of the composition to the selected final media volume.

11. The composition of claim **10** where the bicarbonate ion providing substance sufficient to a produce a bicarbonate ion concentration of between 0.020 M and 0.030 M in the final media volume is 2.1 g of $NaHCO_3$ per liter of final media volume.

* * * * *

EXHIBIT B

US006900056B2

(12) **United States Patent**
Lee et al.

(10) **Patent No.: US 6,900,056 B2**
(45) **Date of Patent: May 31, 2005**

(54) **CHEMICALLY DEFINED MEDIUM FOR CULTURED MAMMALIAN CELLS**

(75) Inventors: **ChiChang Lee**, Norristown, PA (US); **Celia Ly**, Lancaster, PA (US); **Gordon Moore**, Wayne, PA (US); **Robert Perkinson**, Philadelphia, PA (US)

(73) Assignee: **Centocor, Inc.**, Malvern, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 242 days.

(21) Appl. No.: **10/067,382**

(22) Filed: **Feb. 5, 2002**

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(51) **Int. Cl.**[7] **C12N 5/00**
(52) **U.S. Cl.** **435/404**; 435/405; 435/406
(58) **Field of Search** 435/404, 405, 435/406

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,205,126	A	*	5/1980	Cartaya
4,767,704	A	*	8/1988	Cleveland et al.
5,691,203	A	*	11/1997	Katusen et al.
5,814,511	A		9/1998	Chang et al.
6,194,203	B1	*	2/2001	Blum et al.
6,399,381	B1	*	6/2002	Blum et al.

FOREIGN PATENT DOCUMENTS

WO WO 02/101019 A 2 12/2002

OTHER PUBLICATIONS

PCT International Search Report PCT/US02/03274 dated Sep. 18, 2002.

* cited by examiner

Primary Examiner—Leon B. Lankford, Jr.

(57) **ABSTRACT**

The present invention relates to methods and compositions for chemically defined media for growth of mammalian cells for production of commercially useful amounts of expressed proteins.

3 Claims, 2 Drawing Sheets

FIG. 1

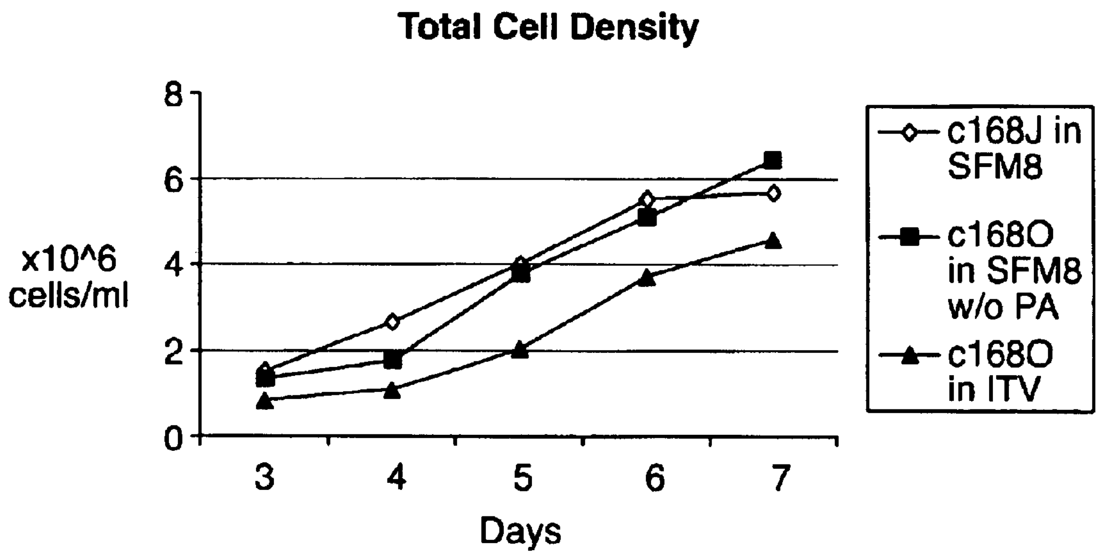

Total Cell Density
x10^6 cells/ml
8
6
4
2
0
3
4
5
6
7
Days
c168J in SFM8
c168O in SFM8 w/o PA
c168O in ITV

FIG. 2

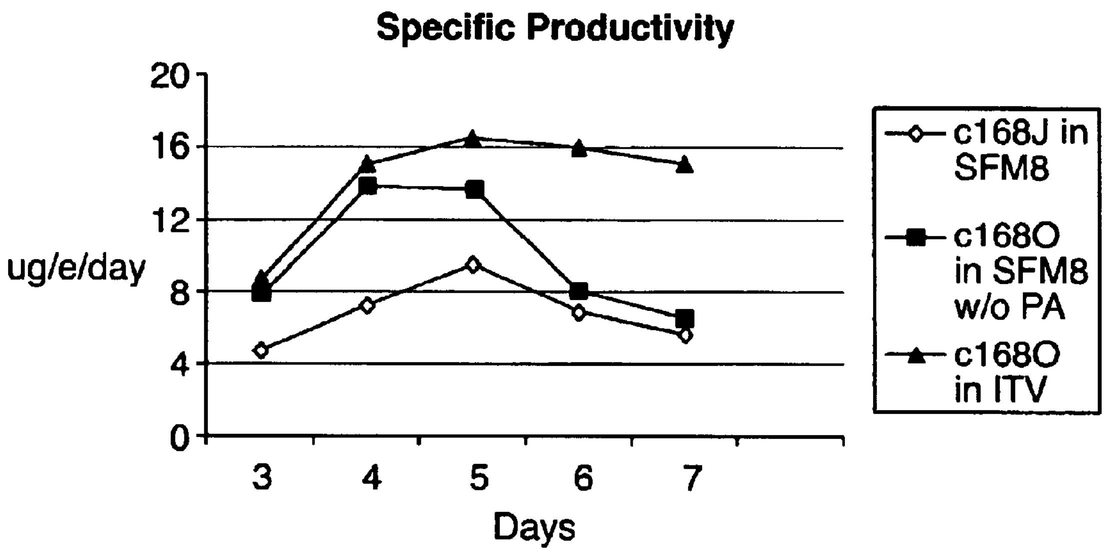

Specific Productivity
ug/e/day
20
16
12
8
4
0
3
4
5
6
7
Days
c168J in SFM8
c168O in SFM8 w/o PA
c168O in ITV

FIG. 3

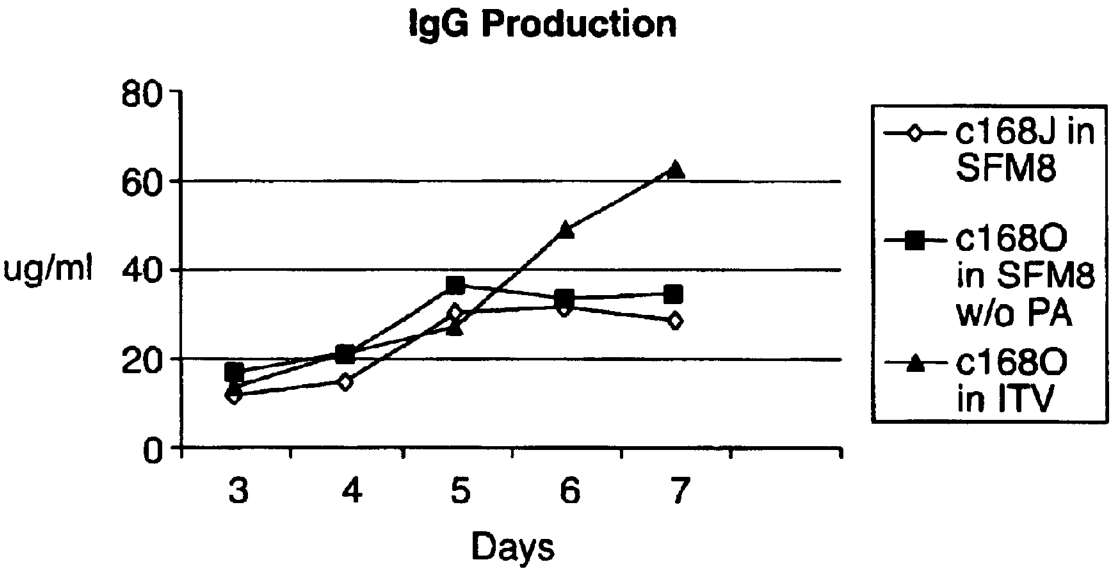
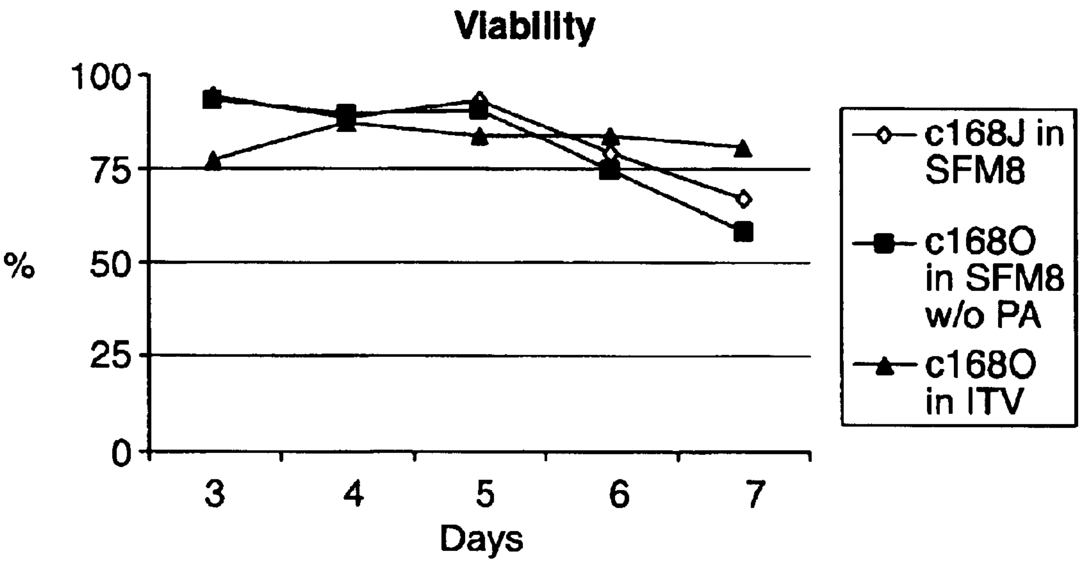

IgG Production
ug/ml
80
60
40
20
0
3
4
5
6
7
Days
c168J in SFM8
c168O in SFM8 w/o PA
c168O in ITV

FIG. 4


Viability
%
100
75
50
25
0
3
4
5
6
7
Days
c168J in SFM8
c168O in SFM8 w/o PA
c168O in ITV

CHEMICALLY DEFINED MEDIUM FOR CULTURED MAMMALIAN CELLS

FIELD OF THE INVENTION

This application is based in part on, and claims priority to, U.S. Provisional No. 60/268,849 filed Feb. 15, 2001, of which is entirely incorporated herein by reference.

The present invention in the field of biotechnology, relates to methods and compositions for providing chemically defined media for growth of cultured mammalian cells for production of commercially useful amounts of expressed proteins.

BACKGROUND OF THE INVENTION

Bovine serum is commonly used in mammalian cell culture to promote cell growth and protein production. Since serum is expensive, non-defined animal materials such as primatone and albumin have been used as serum replacements. However, the quality of these non-defined animal proteins varies from batch to batch and consistent cell growth in these media is difficult to achieve. Moreover, pathogens such as prions and viruses have been identified as potential infectious agents (Balter, M. 2000, Kozak et al. 1996) that may reside in those animal derived products. Many regulations now strongly address these concerns about using serum or non-defined animal proteins in mammalian cells.

To support the growth of animal cells, a variety of components are essential to be included in the culture media. For example, glutamine and glucose are basic energy sources that support animal cell growth. Breakdown of these compounds provides resources for energy-generating pathways, the TCA cycle and glycolysis. The byproducts of these pathways are also the building blocks or sources for bio polymer synthesis (Petch and Bulter 1994). In addition, vitamins, amino acids and growth factors are also essential for robust cell growth by either supressing the cascade of the suicide pathway known as apoptosis or by promoting the progression of the cell cycle so that cells may replicate (Franek F. 1994, Murakami et al. 1982, Mastrangelo et al. 1999, Xie and Wang, 1996, Muhamed Al-Rubeai 1998).

Trace elements are also important for the growth of animal cells. Ham and McKeehan (1979) noticed that adding trace elements, such as Zinc, iron, selenium, copper, molybdenum, and manganese, etc., was important for cloning and continuous passage of animal cells in stringent conditions of serum-free media. Regardless, the importance of supplementing trace elements in the media for animal cells has not been well addressed (Schneider 1989, Merten and Litwin 1991). This may be due to the assumption that trace elements existed as contaminated impurities within serum or non-defined animal derived materials already.

Accordingly, there is also a need to provide chemically defined media for cell culture and/or production of heterologous proteins in commercially useful amounts.

SUMMARY OF INVENTION

The present invention provides chemically defined media (CDF) formulations and methods that provide certain compounds, amino acids, lipids, carbohydrates, trace elements and/or vitamins that provide a chemically defined media that excludes the use of non-defined animal derived raw materials (e.g., but not limited to, primatone, albumin and Excyte™, as well as other similar materials derived from serum or other animal derived proteins or products). Such media compositions and formulations of the present invention allow the growth of myeloma and other cell cultures to provide commercially useful amounts of the desired proteins expressed in such cell cultures. Accordingly the present invention provides specific media, formulations and methods of making and using thereof, as well as proteins provided therefrom. The present invention provides media that provide one or more advantages of being chemically defined, better protein producing, commercially suitable, cost-effective, and/or pose reduced regulatory concerns for proteins produced in cell lines grown therein.

DESCRIPTION OF THE DRAWINGS

FIG. **1** shows by graphical representation that CDM media of the present invention can support high cell density up to 4.5×10^{6} cells/mL on Day 7.

FIG. **2** shows by graphical representation that specific productivity for CDM culture is at 16 μg/10^{6} cells/day.

FIG. **3** shows that at high cell density between 4–5×10^{6} cells/mL, IgG production reached above 60 μg/mL.

FIG. **4** shows by graphical representation that Viability of the CDM spinner culture remained above 75% throughout the experiment.

Also included in FIGS. **1**–**4** are data of C168O in SFM8 without primatone, albumin and C168J in SFM8 as references for comparison.

DETAILED DESCRIPTION

The present invention provides media formulations and methods that provide a chemically defined media that provides advantages over known media, and which can be used for commercial production of mammalian cell-cultured proteins. The present invention also provides a chemically defined media (CDM) comprising novel components, as well as, or optionally further comprising, at least one of specified amino acids, lipids, carbohydrates, trace elements, vitamins, compounds and/or proteins, as described and enabled herein, in combination with what is known in the art.

The present invention avoids of one or more problems associated with media that contains animal derived, or non-defined animal derived, components (e.g., but not limited to, primatone, albumin and excyte, as well as other similar materials derived from serum or other animal proteins in recombinant, synthetic or purified form).

Accordingly, chemically defined media (CDM) compositions and formulations of the present invention allow the growth of myeloma and other cell cultures to provide commercially useful amounts of the desired proteins expressed in such cell cultures. The present invention thus provides specific media formulations that are chemically defined, cost-effective, and pose reduced regulatory concerns compared to known media that comprise animal-derived materials that are not completely defined, or known chemically defined media.

Media of the present invention includes the substitution of specified components, without the use of animal derived proteins. In a preferred embodiment, media of the present invention comprises specified components, e.g., but not limited to, trace elements and vitamins, the media termed "chemically defined media" (CDM). Media of the present invention provides utility and improvements, including, but not limited to, at least one of suitable high cell density growth, improved growth rate, improved growth in scale up, improved viability, improved viability in scale up, improved protein yield, improved protein yield in scale up, and the like.

Suitable cell lines that can be used according to the present invention include any transformed or immortalized mammalian cell line. Such cell lines can include myeloma cell lines, such as Sp2/0, NSO, NSl, CHO, BHK, Ag653, P3X63Ag8.653 cells (ATCC Accession Number CRL-1580) and SP2/0-Ag14 cells (ATCC Accession Number CRL-1851), COS-1 (e.g., ATCC CRL 1650), COS-7 (e.g., ATCC CRL-1651), HEK293, BHK21 (e.g., ATCC CRL-10), CHO (e.g., ATCC CRL 1610, CHO DXB-11, CHO DG44), BSC-1 (e.g., ATCC CRL-26) cell lines, HepG2 cells, P3X63Ag8.653, 293 cells, HeLa cells, NIH 3T3, COS-1, COS-7, NIH 273, and the like, or any cells derived therefrom, including cell fusions of the above, such as to protein producing cells, such as B-cells, antibody producing cells, isolated or cloned spleen or lymph node cells, and the like. A preferred cell line is derived from Sp2/0 and is designated C463A, as described herein.

Cell lines, such as those presented herein, can be adapted to a chemically defined media according to the present invention, using known techniques and/or as described herein. Such methods can take from 1–30 days, or up to several months, depending on the particular cell line and media formulation used. However, adaption of mammalian cells to grow in chemically defined media of the present invention is unexpectedly found to occur in significantly shorter times that known defined or undefined media.

At least one formulation of media of the present invention is unexpectedly discovered to provide at least one of several advantages over known media, including at least one of: (1) supporting improved or robust growth and protein or antibody production from various mammalian cell lines; (2) facilitated adaptation for protein producing cell lines; (3) cost-effective media components, as compared to known components, such as bovine serum and excyte, which do not need to be and are not included; and/or (4) better suitability for regulatory approval as the media components are at least one of better defined, do not include animal derived proteins or other products, and do not contain or potentially contain infectious agents.

The use of this medium in cell culture technologies, such as but not limited to culture dishes, culture plates, culture bottles, suspension culture, spin filter suspension culture, bioreactors, perfusion type bioreactors, mammalian cell fermentation culture, or any other suitable type of cell culture, is also included in the present invention.

A media formulation of the present invention includes at least one of specified buffers, salts, carbohydrates, vitamins, proteins, amino acids, lipids, trace elements, minerals, and the like as described herein in combination with what is known in the art.

The media preferably comprises, in addition to known mammalian or hybridoma cell culture components without undefined protein- or animal-derived components, at least one or more of ammonium metavanadate, cadmium chloride, chromic potassium sulfate, ferric citrate, germanium dioxide, molybdic acid, salt or ammonium salt, nickel sulfate, zirconium chloride and/or hydrocortisone, or any suitable form, salt, halide, hydrate, solution, suspension, emulsion, or colloid thereof, powder and the like. In preferred embodiments, the media comprises, in addition to known components, at least one, two, three, four, five, six, seven, eight, or nine of the above components.

Non-limiting examples of such buffers and include at least one of MOPS, sodium phosphate, potassium phosphate, HEPES, and other known buffers. Salts included in such buffers include, but are not limited to sodium chloride, potassium chloride, and the like. Non-limiting examples are presented as one or more of the salts, as hydrous, anhydrous or other salt form, in the following table:

Component	g/L
INORGANIC SALTS	
AlCl3.6H2O	0.0000001–0.00001
NH4Vo$_3$	0.00000006–0.000001
BaCl$_2$	0.0000002–0.000001
CaCl2.2H2O	0.004–0.09
CoCl2.6H2O	0.0000002–0.00001
CrK(So4)$_2$	0.0000001–0.00001
CuSo4.5H2O	0.0000005–0.00001
FeSo4.7H2O	0.000001–0.0001
Geo$_2$	0.00000005–0.000001
LiCl	0.001–0.1
MgCl.6H2O	0.01–1.0
MnCl(anhyd)	0.00000001–0.000001
Na2Moo4.2H2O	0.00000001–0.000001
NiNo3.6H2O	0.00000002–0.000001
KBr	0.00000001–0.000001
KCl	0.01–1.0
KI	0.00000001–0.000001
RbCl	0.000000001–0.0000001
AgCl	0.0000000004–0.0000001
NaHCo$_3$	0.0000001–0.00001
NaCl	O.1–50
NaF	0.0000004–0.00001
Na2HPo$_4$(anhyd)	0.01–5
Na2Seo$_3$	0.000003–0.0001
SnCl2.2H2O	0.00000001–0.000001
Tio$_2$	0.0000001–0.0001
ZnSo4.7H2O	0.000008–0.0001

Such carbohydrates include, but are not limited to, glucose (dextrose), fructose, mannose, galactose, and any other suitable monosaccharide, disaccharide, polysaccharide, polymer, carbohydrate and the like. Non-limiting examples of amounts include 0.0000001–100 g/L for one or more carbohydrate components.

Such vitamins and co-factors can include, but are not limited to, biotin, ascorbic acid, pantothenate, choline, folate, inositol, niacin, niacinamide, pyridoxal, riboflavin, thamine, cyanocbalamin, L-abscorbic acid and salts, D-biotin, calciferol, choline, cocarboxylase, coenzyme A, 2-deoxyadenosine, 2-deoxyguanosine, 2-deoxycytidine, ergocalciferol, flavin adenosine dinucleotide, FAD, folic acid, D-glucoronic acid, lactone, D-glucoronic acid, glutathione, myo-inositol, mammalian recombinant insulin, menadione, 5'methylcytosine, niacinamide, NADP, NAD, nicotinic acid, oxalacetic acid, p-amino benzoic acid, D-pantothenic acid, pyroxidal, pyroxidine, retinol acetate, riboflavin, α-tocopherol, thiamine, thymidine, UMP, UDP, UTP, AMP, ADP., ATP, GMP, GDP, GTP, CMP, CDP, CTP, TMP, TDP, TTP, vitamin B12, and the like, in any suitable form, such as salt, acid, base, and the like.

Such proteins or amino acids include, but are not limited to, alanine, arginine, asparagine, aspartate, cysteine, cystine, glutamine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and salts or other derivatives thereof. Alternatively, such amino acids include at least one of L-α-amino-n-butyric acid, L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-cysteine, L-citrulline, L-cysteine, D-glucosamine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, hydroxy-L-Proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-omithine, L-phenylalanine, L-proline, L-serine, taurine, L-threonine, L-tryptophan, L-tyrosine, L-valine, and the like, as well as salts, hydrates, hydrides, acids, bases thereof and the like.

Such trace elements and minerals include, but are not limited to, salts (e.g., chlorides, iodides, bromides, fluorides, sodium or potassium salts, phosphates, salts, and the like), acids (e.g., acetates, sulfates, sulfides, nitrates, nitrides, dioxides, and the like), bases (e.g., NaOH, KOH, and the like), of magnesium, potassium, sodium, calcium, and the like, such as sodium acetate, sodium chloride, sodium phosphate, selenium, aluminum, ammonium metavanadate, barium, cadmium, cobalt chloride, chromic potassium sulfate, cupric sulfate, ferric citrate, germanium dioxide, lithium chloride, magnesium chloride, manganese chloride, molybdic acid, nickel nitrate, potassium bromide, potassium iodide, rubidium chloride, silver chloride, sodium fluoride, stannous chloride, sodium silicate, tin chloride tin chloride, titanium chloride, zinc sulfate, zirconium oxychloride, and the like, and salts thereof.

As a further non-limiting example, a formulation of CDM media of the present invention comprises: sodium chloride, 3–5 g/L; potassium chloride, 0.2–0.4 g/L; , HEPES, 5–7 g/L; glucose (dextrose), 3.5–5.5 g/L; biotin, 0.000005–0.000025 g/L; ascorbic acid, 0.002–0.004; pantothenate, 0.002–0.006 g/L; choline, 0.002–0.006 g/L; folate, 0.002–0.006 g/L; inositol, 0.005–0.02 g/L; niacinamide, 0.002–0.006 g/L; pyridoxal, 0.002–0.006 g/L; riboflavin, 0.0002–0.0006 g/L; thiamine, 0.002–0.006 g/L; cyanocbalamin, 0.000005–0.000025 g/L; oxaloacetic acid, 0.1–0.4 g/L; alanine, 0.015–0.035 g/L; asparagine, 0.01–0.035 g/L; arginie, 0.06–0.10 g/L; aspartate, 0.02–0.04 g/L; cysteine, 0.3–0.5 g/L; cystine, 0.05–0.2 g/L; glutamine, 0.8–1.5 g/L; glutamate, 0.06–0.09 g/L; glycine, 0.02–0.04 g/L; histidine, 0.03–0.05 g/L; isoleucine, 0.05–0.25 g/L; leucine, 0.05–0.25 g/L: lysine, 0.05–0.25 g/L; methionine, 0.02–0.04 g/L; phenylalanine, 0.055–0.075. proline, 0.03–0.05 g/L; serine, 0.03–0.055 g/L; threonine, 0.07–0.15 g/L; tryptophan, 0.005–0.025 g/L; tyrosine, 0.05–0.15 g/L; valine, sodium selenate, 0.0000005–0.000060; magnesium sulfate, 0.05–0.2 g/L; potassium chloride, 0.15–0.45 g/L; sodium phosphate, 0.075–0.2 g/L; potassium nitrate, 0.00005–0.00009 g/L; calcium chloride, 0.08–0.25 g/L; sodium pyruvate 0.05–0.4 g/L; insulin, 0.05–2 g/L; hydrocortisone, 20–80 μg/L; linoleic acid, 1–100 mg/L; ethanolamine, 5–25 μg/L; sodium bicarbonate, 1–5 g/L; APO transferrin or ferric citrate, 1–10 mg/L; Pluronic F68, 0.2–2 g/L; sodium hydroxide, 0.3–0.9 g/L; mycophenolic acid, 0.1–2 mg/L; hypoxanthine, 2–5 mg/L; xanthine; 10–200 mg/L; sodium bicarbonate 1.5–4.5 g/L.

Known serum free hybridoma media that can be modified to provide the media of the present invention include, but are not limited to, e.g., Sigma/Aldrich product numbers S2772, S2897 and S8284 (www.sigma-aldrich.com); similar known serum free media include those from Life Technologies, Rockville, Md. (www.lifetech.com) and JRH Biosciences, Lenexa, Kans. (www jrhbio.com). For example, known serum free hybridoma cell cultures can include HEPES or MOPS, sodium bicarbonate, L-glutamine, cholesterol, insulin, BSA, transferrin or ferric citrate, in addition to other serum free mammalian cell culture components. See, e.g., SIGMA catalog, 1998, pp 1776–1777, 1677–1704, 1715–1755, 1795–1847, entirely incorporated herein by reference. Non-limiting examples of known serum free media that can be modified to provide CDM of the present invention include, but are not limited to, sigma media product numbers S2772, S2897 and S8284, as follows:

SIGMA Prod. # Component	S 2897 g/L	S 8284 g/L	S 2772 g/L
INORGANIC SALTS			
AlCl3.6H2O	0.000001	0.000001	0.000001
NH4Vo$_3$	0.0000006	0.0000006	0.0000006
BaCl$_2$	0.000002	0.000002	0.000002
CaCl2.2H2O	0.0441	0.0441	0.0441
CoCl2.6H2O	0.000002	0.000002	0.000002
CrK(So4)$_2$	0.000001	0.000001	0.000001
CuSo4.5H2O	0.0000051	0.0000051	0.0000051
FeSo4.7H2O	0.000834	0.000834	0.000834
Geo$_2$	0.0000005	0.0000005	0.0000005
LiCl	0.01	0.01	0.01
MgCl.6H2O	0.123	0.123	0.123
MnCl(anhyd)	0.0000001	0.0000001	0.0000001
Na2Moo4.2H2O	0.0000001	0.0000001	0.0000001
NiNo3.6H2O	0.0000002	0.0000002	0.0000002
KBr	0.0000001	0.0000001	0.0000001
KCl	0.224	0.224	0.224
KI	0.0000001	0.0000001	0.0000001
RbCl	0.00000001	0.00000001	0.00000001
AgCl	0.0000000044	0.0000000044	0.0000000044
NaHCo$_3$	—	2.25	2.25
NaCl	7.599	7.599	7.599
NaF	0.000004	0.000004	0.000004
Na2HPo$_4$(anhyd)	0.39739	0.39739	0.39739
Na2Seo$_3$	0.00003	0.00003	0.00003
SnCl2.2H2O	0.0000001	0.0000001	0.0000001
Tio$_2$	0.000001	0.000001	0.000001
ZnSo4.7H2O	0.000863	0.000863	0.000863
AMINO ACIDS			
L-Alanine	0.009	0.009	0.009
L-Arginine	0.211	0.211	0.211
L-Asparagine.H2O	0.03401	0.03401	0.03401
L-Aspartic Acid	0.0133	0.0133	0.0133
L-Citrulline	0.005	0.005	0.005
L-Cysteine.HCl.H2O	0.035	0.035	0.035
L-Glutamic Acid	0.0147	0.0147	0.0147
L-Glutamine	0.396	0.396	0.396
Glycine	0.00751	0.00751	0.00751
L-Histidine.HCl.H2O	0.071	0.071	0.071
L-Isoleucine	0.164	0.164	0.164
L-Leucine	0.133	0.133	0.133
L-Lysine.HCl	0.109	0.109	0.109
L-Methionine	0.015	0.015	0.015
L-Ornithine	0.008	0.008	0.008
L-Phenylalanine	0.055	0.055	0.055

See, e.g., Ham et al., Proc. Natl. Acad. Sci. USA 53: 288–193 (1965); Myoken etal., In Vitro 25: 477–480 (1989).

More preferably, the media further comprises at least one selected from the group consisting of buffers, salts, carbohydrates, amino acids, lipids, vitamins, co-factors, and the like in suitable form. Suitable media that can be modified according to the present invention can include one or more or a combination of Iscove's modified media, Dulbecco's Modified Eagle Medium, Ham's F-12 media, e.g., as provided by SIGMA, LIFE TECHNOLOGIES OR JRH

BIOSCIENCES, as listed above. Non-limiting examples, include, but are not limited to:

Iscove's Modified Media: (Sigma I2510, I7633, I2762, I3390):		
SIGMA Prod. Num. COMPONENT	I2510, I7633 g/L	I2762, I3390 g/L
INORG. SALTS		
CaCl2.2H2O	0.219	0.219
MgSO4 (anhyd)	0.09767	0.09767
KCl	0.33	0.33
KNO3	0.000076	0.000076
NaHCO3	—	3.024
KCl	4.505	4.505
NaH2PO4 (anhyd.)	0.109	0.109
Na2SeO3	0.000017	0.000017
AMINO ACIDS		
Alanine	0.025	0.025
L-Arginine.HCl	0.084	0.084
L-Asparagine.H_2O	0.0284	0.0284
L-Aspartic Acid	0.03	0.03
L-Cystine.2HCl	0.09124	0.09124
L-Glutamic Acid	0.075	0.075
L-Glutamine	0.584	—
Glycine	0.03	0.03
L-Histidine.HCl.H_2O	0.042	0.042
L-Isoleucine	0.105	0.105
L-Leucine	0.105	0.105
L-Lysine.HCl	0.146	0.146
L-Methionine	0.03	0.03
L-Phenylalanine	0.066	0.066
L-Proline	0.04	0.04
L-Serine	0.042	0.042
L-Threonine	0.095	0.095
L-Tryptophan	0.016	0.016
L-Tyrosine.2Na.2H2O	0.10379	0.10379
L-Valine	0.094	0.094
VITAMINS		
D-Biotin	0.000013	0.000013
Choline Chloride	0.004	0.004

-continued

Iscove's Modified Media: (Sigma I2510, I7633, I2762, I3390):		
SIGMA Prod. Num. COMPONENT	I2510, I7633 g/L	I2762, I3390 g/L
Folic Acid	0.004	0.004
myo-Inositol	0.0072	0.0072
Niacinamide	0.004	0.004
D-Pantothenic Acid.½Ca	0.0004	0.004
Pyridoxal.HCl	0.004	0.004
Riboflavin	0.0004	0.0004
Thiamine.HCl	0.004	0.004
Vitamin B12	0.000013	0.000013
OTHER		
D-Glucose	4.5	4.5
HEPES	5.958	5.958
Phenol Red.Na	0.016	0.016
Pyruvic Acid.Na	0.11	0.11
ADD		
NaHCO3	3.024	—
L-Glutamine	—	0.584
Grams of powder required to prepare 1 L	17.7	N/A

See, e.g., Iscove et al., J. Exp. Med. 147: 923–933 (1978); Iscove, et al., Exp. Cell Res. 126: 121–126 (1980).

Dulbecco's Modified Eagle's Medium (e.g., Sigma D0422, D1152, D2429, D2554, D2902, D3656, D5030, D5280, D5523).									
SIGMA Prod # COMPONENT	D0422 g/L	D1152 g/L	D2429 g/L	D2554 g/L	D2902 g/L	D3656 g/L	D5030 g/L	D5280 g/L	D5523 g/L
INORGANIC SALTS									
CaCl2.2H2O	0.265	0.265	2.65	2.65	0.265	0.265	0.265	0.265	0.265
Fe(NO3)3.9H2O	0.0001	0.0001	0.001	0.001	0.0001	0.0001	0.0001	0.0001	0.0001
MgSO4	0.09767	0.09767	0.9767	0.9767	0.09767	0.09767	0.09767	0.09767	0.09767
KCl	0.4	0.4	4.0	4.0	0.4	0.4	0.4	0.4	0.4
NaHCO3	3.7	—	—	—	—	—			
NaCl	6.4	4.4	64.0	64.0	6.4	6.4	6.4	6.4	6.4
NaH2PO4	0.109	0.109	1.09	1.09	0.109	—	0.109	0.109	0.109
Succinic Acid		—	—	—	—	—	0.075	—	
Sodium Succinate		—	—	—	—	—	0.1	—	
AMINO ACIDS									
L-Arginine.HCl	0.84	0.084	0.84	0.84	0.084	0.084	0.084	0.084	0.084
L-Cystine.2HCl	—	0.0626	0.626	0.626	0.0626	0.0626	0.0626	0.0626	0.0626
L-Glutamine	0.03	0.584	0.30	0.30	0.584	0.584	—	—	0.584
Glycine	0.042	0.030	0.42	0.42	0.030	0.030	0.030	0.030	0.030
L-Histidine.HCl.H2O	0.105	0.042	1.05	1.05	0.042	0.042	0.042	0.042	0.042
L-Isoleucine	0.105	0.105	1.05	1.05	0.105	0.105	0.105	0.105	0.105
L-Leucine	1.46	0.105	1.46	1.46	0.105	0.105	0.105	0.105	0.105

-continued

Dulbecco's Modified Eagle's Medium (e.g., Sigma D0422, D1152, D2429, D2554, D2902, D3656, D5030, D5280, D5523).

SIGMA Prod # COMPONENT	D0422 g/L	D1152 g/L	D2429 g/L	D2554 g/L	D2902 g/L	D3656 g/L	D5030 g/L	D5280 g/L	D5523 g/L
L-Lysine.HCl	—	0.146	0.30	0.30	0.146	0.146	0.146	0.146	0.146
L-Methionine	0.066	0.030	0.66	0.66	0.030	0.030	0.030	0.030	0.030
L-Phenylalanine	0.042	0.066	0.42	0.42	0.066	0.066	0.066	0.066	0.066
L-Serine	0.095	0.042	0.95	0.95	0.042	0.042	0.042	0.042	0.042
L-Threonine	0.016	0.095	0.16	0.16	0.095	0.095	0.095	0.095	0.095
L-Tryptophan	0.016	—	—	0.016	0.016	0.016	0.016	0.016	
L-Tyrosine (free base)	0.10379	—	—	1.0379	1.0379	—	—	0.072	—
L-Tyrosine.2Na.2 H2O	0.10379	0.10379	0.10379	0.10379	—	0.10379			
L-Valine	0.094	0.094	0.94	0.94	0.094	0.094	0.094	0.094	0.094
VITAMINS									
Choline Bitartrate	0.004	—	0.04	0.04	—	—	—	0.0072	—
Choline Chloride	0.004	0.004	—	—	0.004	0.004	0.004	—	0.004
Folic Acid	0.0072	0.004	0.072	0.072	0.004	0.004	0.004	0.004	0.004
myo-Inositol	0.004	0.0072	0.04	0.04	0.0072	0.0072	0.0072	0.0072	0.0072
Niacinamide	0.004	0.004	0.04	0.04	0.004	0.004	0.004	0.004	0.004
D-Pantothenic Acid.½Ca	0.004	0.004	—	—	0.004	0.004	0.004	0.004	0.004
Pyridoxal.HCl	—	0.004	0.04	0.04	0.004	0.004	0.004	0.004	0.004
Pyridoxine.HCl	0.0004	—	0.004	0.004	—	—	—	—	—
Riboflavin	0.004	0.0004	0.04	0.04	0.0004	0.0004	0.0004	0.0004	0.0004
Thiamine.HCl	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
OTHER									
D-Glucose	4.5	4.5	10.0	45.0	1.0	4.5	—	1.0	—
HEPES	—	5.958	—	—	—	—	—	—	0.0159
Phenol Red.Na	0.0159	0.0159	0.159	0.159	—	0.0159	—	0.0093	0.11
Pyruvic Acid.Na	0.11	—	1.1	1.1	0.11	—	—	0.11	—
ADD									
Glucose	—	—	—	—	—	1.0	—	—	—
L-Glutamine	0.584	—	0.584	0.584	—	—	0.584	0.584	—
L-Cystine.2HCl	—	—	—	—	—	—	—	—	—
L-Leucine	—	—	—	—	—	—	—	—	—
L-Lysine.HCl	—	—	—	—	—	—	—	—	—
L-Methionine	—	—	—	—	—	—	—	—	—
NaHCO3	—	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7
NaH2PO4	—	—	—	—	—	0.109	—	—	—
Phenol Red.Na	—	—	—	—	—	—	—	—	—
Pyruvic Acid.Na	—	—	—	—	—	—	—	—	—
Grams of powder to prepare 1 L	N/A	17.4	N/A	N/A	N/A	N/A	N/A	N/A	10.0

See, e.g., Dulbecco and Freeman, Virology 8: 396–397 (1959); Smith et al.,, J. D. Freeman, G., Vogt, M. and Dulbecco, R. (1960). Virology 12: 185–196 (1960); Morton, In Vitro 6: 89 (1970); Rutzky and Pumper, In Vitro 9: 468 (1974).

Ham's F-12/Dulbecco's Modified Eagle's Medium (e.g., Sigma D6905, D8900, D2906, D9785, D6421)

SIGMA Prod.# COMPONENT	D6905, D8900 g/L	D2906 g/L	D9785 g/L	D6421 g/L
INORGANIC SALTS				
$CaCl_2.2H_2O$	0.1545	0.1545	—	0.1545
$CuSO4.5H_2O$	0.0000013	0.0000013	0.0000013	0.0000013
$Fe(NO3)3.9H_2O$	0.00005	0.00005	0.00005	0.00005
$FeSO_4.7H_2O$	0.000417	0.000417	0.000417	0.000417

-continued

Ham's F-12/Dulbecco's Modified Eagle's Medium (e.g., Sigma D6905, D8900, D2906, D9785, D6421)

SIGMA Prod.# COMPONENT	D6905, D8900 g/L	D2906 g/L	D9785 g/L	D6421 g/L
$MgCl.6H_2O$	0.06120	0.0612	—	0.0612
$MgSO_4$	0.04884	0.04884	—	0.04884
KCl	0.3118	0.3118	0.3118	0.3118
$NaHCO_3$	—	—	—	1.2
NaCl	6.996	6.996	6.996	6.996
Na2HPO4	0.07102	0.07102	0.07102	0.07102
NaH2PO4	0.0543	0.0543	0.0543	0.0543

-continued

Ham's F-12/Dulbecco's Modified Eagle's Medium (e.g., Sigma D6905, D8900, D2906, D9785, D6421)

SIGMA Prod.# COMPONENT	D6905, D8900 g/L	D2906 g/L	D9785 g/L	D6421 g/L
ZnSO4.7H2O	0.000432	0.000432	0.000432	0.000432
AMINO ACIDS				
L-Alanine	0.00445	0.00445	0.00445	0.0045
L-Arginine.HCl	0.1475	0.1475	0.1475	0.1475
L-Asparagine.H2O	0.0075	0.0075	0.0075	0.0075
L-Aspartic Acid	0.00665	0.00665	0.00665	0.00665
L-Cystine.HCl.H2O	0.01756	0.01756	0.01756	0.01756
L-Cysteine.2HCl	0.03129	0.03129	0.03129	0.03129
L-Glutamic Acid	0.00735	0.00735	0.00735	0.00735
L-Glutamine	0.365	0.365	—	—
Glycine	0.01875	0.01875	0.01875	0.01875
L-Histidine.HCl.H_2O	0.03148	0.03148	0.03148	0.03148
L-Isoleucine	0.05447	0.05447	0.05447	0.5447
L-Leucine	0.05905	0.05905	—	0.05905
L-Lysine.HCl	0.09125	0.09125	—	0.09125
L-Methionine	0.01724	0.01724	—	0.01724
L-Phenylalanine	0.03548	0.03548	0.03548	0.03548
L-Proline	0.01725	0.01725	0.01725	0.01725
L-Serine	0.02625	0.02625	0.02625	0.02625
L-Threonine	0.05345	0.05345	0.05345	0.05345
L-Tryptophan	0.00902	0.00902	0.00902	0.00902
L-Tyrosine.2Na.$2H_2O$	0.05579	0.05579	0.05579	0.05579
L-Valine	0.05285	0.05285	0.05285	0.05285
VITAMINS				
D-Biotin	0.0000035	0.0000035	0.0000035	0.0000035
Choline Chloride	0.00898	0.00898	0.00898	0.00898
Folic Acid	0.00265	0.00266	0.00266	0.00266
myo-Inositol	0.0126	0.0126	0.0126	0.0126
Niacinamide	0.00202	0.00202	0.00202	0.00202
D-Pantothenic Acid.½Ca	0.00224	0.00224	0.00224	0.00224
Pyridoxal.HCl	0.002	0.002	0.002	—
Pyridoxine.HCl	0.000031	0.000031	0.000031	0.002031
Riboflavin	0.000219	0.000219	0.000219	0.000219
Thiamine.HCl	0.00217	0.00217	0.00217	0.00217
Vitamin B-12	0.00068	0.00068	0.00068	0.00068

See, e.g., Barnes and Sato, Analyt. Biochem. 102:255–270 (1980).

Any suitable or desired protein for mammalian cell expression can be used in cell culture using media according to the present invention. Non-limiting examples of such proteins include, but are not limited to therapeutic or diagnostic proteins, such as eukaryotic or prokaryotic proteins. Preferred proteins can include, but art not limited to, cytokines, receptors, soluble receptors, interleukins, growth factors, and the like.

Citations

All publications or patents cited herein are entirely incorporated herein by reference as they show the state of the art at the time of the present invention and/or to provide description and enablement of the present invention. Publications refer to scientific or patent publications, or any other information available in any media format, including all recorded, electronic or printed formats. The following references are entirely incorporated herein by reference: Ausubel, et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y. (1987–1999); Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2^{nd} Edition, Cold Spring Harbor, N.Y. (1989); Harlow and Lane, Antibodies, a Laboratory Manual, Cold Spring Harbor, N.Y. (1989); Colligan, et al., eds., Current Protocols in Immunology, John Wiley & Sons, Inc., N.Y. (1994–1998); Colligan et al., Current Protocols in Protein Science, John Wiley & Sons, NY, N.Y., (1997–1999).

EXAMPLES

Example 1

Production of Protein in Chemically Defined Media of the Present Invention Using Adapted Cell lines

An IgG protein producing myeloma cell line named C168O was not ideally suited for commercial production of IgG or for suitable regulatory approval, due to need for media components of known serum free media containing animal protein derived preparations that were not sufficiently defined or characterized, such as Excyte and others. This Excyte dependency was not able to be alleviated by adding chemically defined lipids or other components. However, when Excyte was removed and trace elements/vitamins were supplemented, a robust growth of C168O was achieved. This medium without primatone, albumin and excyte but supplemented with trace elements and vitamins is now called "CDM". A semi-batch culture of C168O in CDM medium showed that CDM medium was able to support both high cell density growth and high IgG production.

Another myeloma cell line called C463A is capable of growing in various commercial defined media. However, this growth was not ideally suited for commercial production of IgG or for suitable regulatory approval. C463A is derived from Sp2/0 and potentially can be used as a transfection host to develop commerically suitable cell lines. In semi-batch cultures, the cell density of C463A in CDM medium of the present invention routinely reached 6 to 7 million cells per milliliter (ml) compared with 3 to 4 million per ml in other tested defined media. The viability is similar amongst all tested media (80% to 90%). Apparently, CDM has the capability to support cell growth at a higher density than other chemically defined media.

To adapt cell lines derived from Sp2/0 cells in chemically defined media is a lengthy process. It usually takes several months to one year to obtain one. When CDM medium is used, we noticed that the length of time for adaptation was much shorter than that in other defined media. In one case, it took only a few weeks to obtain CDM culture compared to several months from previous experiences.

In summary, we find that trace elements and vitamins are essential for the growth of myeloma cells in the absence of bovine serum and non-defined animal derived materials. A chemically defined formulation was generated based on the addition of trace elements and vitamins to a suitable serum free media system. This formulation provides several advantages: 1. Supports robust growth and IgG or other protein production of various myeloma and other cell lines, 2. Easy adaptation for mammalian cells, e.g., Sp2/0-derived IgG or protein producing cell lines, 3. Cost-effective since expensive components, such as bovine serum and excyte, are excluded and 4. Regulatory-friendly since potentially infectious agents are eliminated.

The use of this medium in perfusion type bioreactors is or other types of cell culture can also be used according to the present invention.

Formulation of CDM Medium:

The formulation of a CDM media of the present invention is provided as follows, e.g., Tables A-B. Table A1 shows the components added to make the media. Tables A2–A3 and B1–B4 show the listing of components for the additional formulations used in Table A1. The components are available from commercial sources, as individual components, or as custom formulations that can be ordered, e.g., from Sigma (St. Louis, Mo., USA), Aldrich (St. Louis, Mo., USA), JRH Biosciences (Lenexa, Kans., USA), and the like.

Tables A1–A3:

TABLE A1

NON-LIMITING EXAMPLE OF CDM MEDIA OF THE INVENTION

Components	Final Concentration In Media	Physical Property	Storage Temp.
CM-2 (Part A, Table A2)	18.8 g/l	Powder	+2–8° C.
CH-2 (Part B; Table A3)	10 ml/l	Liquid	−20° C.
$NaHCO_3$	3.02 g/l	Powder	Ambient
Bovine APO	5 mg/l	Stock solution	+2–8° C.
Transferrin or ferric citrate		Powder	+2–8° C.
Pluronic F68	0.8 g/l	Stock Solution	+2–8° C.
		Powder	Ambient
		Powder	+2–8° C.
NaOH	0.7 g/l	Stock Solution	Ambient
		Pellets	Ambient
Ethanolamine	10 or 20 mg/l	Stock Solution	−20° C.
		Liquid	Ambient
Glutamine	0.29 g/l	Powder	Ambient
Mycophenolic acid	0.5 mg/l	Stock Solution WSS	+2–8° C.
Hypoxanthine	2.5 mg/l	2	
Xanthine (MHX)	50 mg/l		
Hydrocortisone	20 ug/l	WSS 9	+2–8° C.
Vitamins (Table B1)	1X	100X Liquid	+2–8° C.
Trace Minerals 1 (Table B1)	1X	1000X Liquid	+2–8° C.
Trace Minerals 2 (Table B2)	1X	1000X Liquid	+2–8° C.
Trace Minerals 3 (Table B3)	1X	1000X Liquid	+2–8° C.

Preparation Instructions: Add components in order listed above. The sodium hydroxide should be made the same day.
Note: Prior to pH adjustment, pH = 6.7–6.8. The density at liquid stock solutions are the same as water (p-1 g/ml). Therefore, volume or weight can be used alternatively.

S1 pH: 7.3–7.6
S1 Osm: 305–368

TABLE A2

CM-2 (Part A)

Component	Final Conc. gm/L (Dry, 18.8 g total/L)
Sodium Chloride	4.505
Potassium Chloride	0.330
Sodium Phosphate Monobasic H_2O	0.125
Magnesium Sulfate, Anhydrous	0.09767
Potassium Nitrate	0.000076
Sodium Selenite	0.0000173
Calcium Chloride, Anhydrous	0.165
L-Alanine	0.025
L-Asparagine H_2O	0.0284
L-Arginine HCl	0.084
L-Aspartic Acid	0.030
L-Cysteine HCl H_2O	0.4175
L-Cystine 2HCl	0.09124
L-Glutamic Acid	0.075
L-Glutamine	0.8763
Glycine	0.030
L-Histidine HCl H_2O	0.042
L-Isoleucine	0.105

TABLE A2-continued

CM-2 (Part A)

Component	Final Conc. gm/L (Dry, 18.8 g total/L)
L-Leucine	0.105
L-Lysine HCl	0.146
L-Methionine	0.030
L-Phenylalanine	0.066
L-Proline	0.040
L-Serine	0.042
L-Threonine	0.095
L-Tryptophan	0.016
L-Tyrosine 2Na $2H_2O$	0.10379
L-Valine	0.094
Dextrose	4.500
Sodium Pyruvate	0.220
Biotin	0.000013
Ascorbic Acid	0.003
D-Ca Pantothenate	0.004
Choline Chloride	0.004
Folic Acid	0.004
i-Inositol	0.0072
Niacinamide	0.004
Pyridoxal HCl	0.004
Riboflavin	0.0004
Thiamine HCl	0.004
Cyanocobalamin	0.000013
Oxalacetic Acid	0.300
HEPES	5.958
	18.7776193

TABLE A3

CH-2 (Part B) (100X)

Component	100X: Amount/L	Final Conc.
Insulin	1.0 g	10 mg/L
Hydrocortisone	200 μg	2 μg/L
Linoleic Acid	500 mg	5 mg/L
Ethanolamine (1.02 mg/μl)	1020 g	10 mg/L
NaCl	8.5 g	85 mg/L

CDM medium is prepared according to Table A1 by adding components CH-2, parts A (18.8 gm/L, Table A2) and B (10 ml/L (100×), Table A3), followed by $NaHCO_3$ (3.02 g/L), Bovine APO transferrin or ferric citrate (5 mg/L), Pluronic F68 (0.8 g/L), NaOH (0.7 g/L), Ethanolamine (10 μl/L), Glutamine (0.29 g/L), mycophenolic acid (0.5 mg/L), hypoxanthine (2.5 mg/L), xanthine (50 mg/L), hydrocortisone (20 μg/L), vitamins (100×, 10 ml/L, Table B 1), trace minerals 1 (1000×, 0.33–1.0 ml/L, Table B2), trace minerals 2 (1000×, 0.33–1.0 ml/L, Table B3), trace minerals 3 (1000×, 0.33–1.0 ml/L, Table B4). In this example of CDM medium of the present invention, the working concentration of trace elements is 0.33–1.00×, and 1× for vitamins.

Tables B1–B4:

TABLE B1

Vitamin Solution (100X)		
Component	Final Concentration Added(1X) mg/L	Liquid 100X mg/L
NaCl	85.00	8500.00
D-Calcium Pantothenate	1.00	100.00
Choline Chloride	1.00	100.00
Folic Acid	1.00	100.00
i-Inositol	2.00	200.00
Nicotinamide	1.00	100.00
Pyridoxine-HCl	1.00	100.00
Riboflavin	0.1	10.00
Thiamine-HCl	1.00	100.00

TABLE B2

Trace Metals 1, 1000X			
COMPONENT	Final Conc. Added 0.33X μg/L	Final Conc. Added 1X μg/L	Liquid 1000X mg/L
$CuSO_4$—$5H_2O$	0.53	1.59	1.60
$ZnSO_4$,—$7H_2O$	284.8	854.4	863.00
Selenite-2Na	5.7	17.1	17.30
Ferric Citrate	381.2	1143.6	1155.10

TABLE B3

Trace Metals 2, 1000X			
COMPONENT	Final Concentration Added (0.33X) μg/L	Final Concentration Added (1X) μg/L	Liquid 1000X mg/L
$AlCl_4$, $6H_2O$	0.40	1.20	1.20
$AgNO_3$	0.056	0.168	0.17
Ba $(C_2H_4O_2)_2$	0.842	2.53	2.55

TABLE B3-continued

Trace Metals 2, 1000X			
COMPONENT	Final Concentration Added (0.33X) μg/L	Final Concentration Added (1X) μg/L	Liquid 1000X mg/L
KBr	0.040	0.12	0.12
$CdCl_2$	0.75	2.25	2.28
$CoCl_26H_2O$	0.785	2.355	2.38
$CrCl_2$, (anhydeous)	0.015	0.045	0.32
NaF	1.39	4.17	4.20
GeO_2	0.175	0.525	0.53
K1	0.056	0.168	0.17
RbCl	0.400	1.20	1.21
$ZrOCl_2$ $8H_2O$	1.06	3.18	3.22

TABLE B4

Trace Metals 3, 1000X			
COMPONENT	Final Concentration Added (0.33X) μg/L	Final Concentration Added (1X) μg/L	Liquid 1000X mg/L
$MnSO_4$ H_2O	0.056	0.168	0.17
$NaSiO_3$ $9H_2O$	46.2	138.6	140.00
Molybdic Acid, Ammonium Salts	0.409	1.227	1.24
NH_4 VO_3	0.21	0.63	0.65
$NiSO_4$ $6H_2O$	0.043	0.129	0.13
$SnCl_2$ (anhydrous)	0.040	0.120	0.12

In this experiment, a chemically defined commercial medium, CD-hybridoma, from Gibco/Life Technology was used as a reference medium. A semi-batch growth profile (a 75% media change was performed daily after Day 3 of the experiment) was initiated to determine the effects of various additives on CDM media. Data at Day 5 were used for this comparison.

TABLE I

Comparing the effects of various additives on CDM w/o PAE in a Semi-Batch Growth Profile of C463A.				
Cultures	Media Identification	Viable Cell Density (e/mL)	Total Cell Density (e/mL)	% Viability
A	CDM w/o OPI (oxaloacetate, pyruvate and insulin), bovine transferrin or ferric citrate and defined lipids, trace elements and vitamins	3.24	5.39	60
B	CDM in A above + OPI (oxaloacetate, pyruvate and insulin)	1.14	1.90	60
C	CDM in A above + bovine transferrin or ferric citrate and defined lipids	3.32	4.68	71
D	CDM in A above + trace elements and vitamins (CDM)	5.22	6.54	80
E	CDM in A above + OPI + transferrin or ferric citrate and defined lipids	1.68	2.26	74

TABLE I-continued

Comparing the effects of various additives on CDM w/o PAE in a Semi-Batch Growth Profile of C463A.

Cultures	Media Identification	Viable Cell Density (e/mL)	Total Cell Density (e/mL)	% Viability
F	CDM in A above + OPI + transferrin or ferric citrate + lipids + trace elements + vitamins	2.74	3.74	73
G	CDM in A above + 1% Sigma PFSF	3.6	4.72	76
H	LTI's CD Hybridoma media-modified	2.64	3.84	69
I	LTI's CD Hybridoma media-modified	3.34	5.04	66

CDM Medium Performs Best Compared to Other Tested Chemically Defined Media

Another semi-batch growth profile experiment was initiated to compare the growth performance of C463A in CDM medium to other commercial chemically defined media. Day 3 and subsequent media change is similar to that stated in Table I.

Table II shows the results collected on Day 5 of the semi-batch experiment. The CDM culture reached the highest viable and total densities among the group. C463A viability in CDM medium was also the highest of the four cultures at 82%. The outcome of this experiment reveals that CDM medium still provides the best support for C463A growth.

TABLE II

Comparing CDM to other chemically defined commercial media in a semi-batch growth profile of C463A

Cultures	Media Identification	Viable Cell Density (e/mL)	Total Cell Density (e/mL)	% Viability
A	CDHY + Sigma S8284 PFSF (50:50)	2.47	3.45	72
B	CDHY + trace elements + vitamins	1.58	3.45	46
C	CDM including + trace elements + vitamins (CDM)	3.86	4.71	82
D	LTI's CD Hybridoma media-modified	1.52	4.03	38

CDM Medium supports high cell density growth and IgG production of C168O, a Remicade producing cell line.

Once CDM medium was determined to enhance growth in our new host cell line, a semi-batch experiment in spinners was initiated for C168O, a Remicade producing cell line derived from C168J (see, e.g.,. FIG. A shows that CDM can support high cell density up to 4.5×10^6 cells/mL on Day 7. In FIG. B, specific productivity for CDM culture is at 16 ug/10^6 cells/day. FIG. C shows that at high cell density between 4–5×10^6 cells/mL, IgG production reached above 60 ug/mL. Viability of the CDM spinner culture remained above 75% throughout the experiment as seen in FIG. D.

Quick Adaptation in CDM Medium

Previously, the adaptation of myeloma cell lines to defined media has been difficult and may take up to 1 year to complete. With CDM medium, the adaptation period has decreased to several weeks. Below, Table III shows another IgG-producing cell line, C380C, adapted to CDM medium in a short period of about 4–5 weeks. After C380C sustained stability in CDM medium, viability was maintained above 90% and doubling time remained within 30–35 hrs. The specific productivity and overgrown IgG titer of C380C in CDM are above that when grown in IMDM w/ 5% FBS medium.

TABLE III

Adaptation of C380C culture in CDM medium

Media	Viability	Mean Doubling Time	Specific Productivity (ug/e/day)	Spent culture IgG titer (ug/mL)
IMDM w/ 5% FBS	>90%	~22–24 hrs.	12–13	55–95

TABLE III-continued

Adaptation of C380C culture in CDM medium

Media	Viability	Mean Doubling Time	Specific Productivity (ug/e/day)	Spent culture IgG titer (ug/mL)
CDM	>90%	Now: 30–40 hrs.	17–22	75–140

REFERENCES

Balter, M. 2000, On the hunt for a wolf in sheep's clothing. Science 287: 1906–1908

Franek, F. (1994) Starvation-Induced Programmed Death of Hybridoma Cells: Prevention by Amino Acid Mixtures. Biotechnology and Bioengineering. 45: 86–90.

Ham, R. G., and Mckeehan, W. L., 1979, Media and growth requirements, Mehhods Enzymol 58:44–93

Kozak R. W., C. F. Golker, P. Stadler, 1996, Transmissible spongiform encephalopathies : Minimizing the risk of transmission by biological/biopharmaceutical products: an industry perspective. Dev. Biol. Stand 88:257–264

Mastrangelo A. J., Shifa Zou, J. M. Hardwick, M. J. Betenbough, 1999, Antiapoptosis Chemicals Prolong Productive Lifetimes of Mammalian Cells upon Sindbis Virus Vector Infection. Biotechnology and Bioengineering. 65: 298–305.

Merten, O. -W., J. Litwin, 1991, Serum-free medium for fermentor cultures of hybridomas. Cytotechnology. 5: 69–82.

Mohamed Al-Rubeai, 1998, Apoptosis and cell cycle technology in Advances Biochemical Engineering/ Biotechnology. Vol.69 pp 225–249, Springer-Verlag Berlin Heidelberg

Murakami, H., H. Masui, G. H. Sato, N. Sueoka, T. P. Chow, and T. Kano-Sueoka, 1982, Growth of hybridoma cells in se serum-free medium: Ethanolamine is an essential component. Proc. Natl. Acad. Sci. USA. 79: 1158–1162.

Petch, D. and M. Butler, 1994, Profile of Energy Metabolism in a Murine Hybridoma: Glucose and Glutamine Utilization. Journal of Cellular Physiology. 161: 71–76.

Schneider, Yves-Jacques. (1988) Optimisation of hybridoma cell growth and monoclonal antibody secretion in a chemically defined serum- and protein-free culture medium. Journal of Immunological Methods. 116: 65–77.

Xie, Liangshi and Daniel I. C. Wang, 1996, High Cell Density and High Monoclonal Antibody Production Through Medium Design and Rational Control in a Bioreactor. Biotechnology and Bioengineering. 51: 725–729.

It will be clear that the invention can be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

What is claimed is:

1. A chemically defined medium, said medium suitable for adaptation and growth of immortalized mammalian cells in culture to high cell densities, said medium comprising, sodium chloride, 3–5 g/L; potassium chloride, 0.2–0.4 g/L; , HEPES, 5–7 g/L; glucose (dextrose), 3.5–5.5 g/L; biotin, 0.000005–0.000025 g/L; ascorbic acid, 0.002–0.004 g/L; pantothenate, 0.002–0.006 g/L; choline, 0.002–0.006 g/L; folate, 0.002–0.006 g/L; inositol, 0.005–0.02 g/L; niacinamide, 0.002–0.006 g/L; pyridoxal, 0.002–0.006 g/L; riboflavin, 0.0002–0.0006 g/L; thiamine, 0.002–0.006 g/L; cyanocbalamin, 0.000005–0.000025 g/L; oxaloacetic acid, 0.1–0.4 g/L; alanine, 0.015–0.035 g/L; asparagine, 0.01–0.035 g/L; arginine, 0.06–0.10 g/L; aspartate, 0.02–0.04 g/L; cysteine, 0.3–0.5 g/L; cystine, 0.05–0.2 g/L; glutamine, 0.8–1.5 g/L; glutamate, 0.06–0.09 g/L; glycine, 0.02–0.04 g/L; histidine, 0.03–0.05 g/L; isoleucine, 0.05–0.25 g/L; leucine, 0.05–0.25 g/L; lysine, 0.05–0.25 g/L; methionine, 0.02–0.04 g/L; phenylalanine, 0.055–0.075 g/L; proline, 0.03–0.05 g/L; serine, 0.03–0.55 g/L; threonine, 0.07–0.15 g/L; tryptophan, 0.005–0.025 g/L; tyrosine, 0.05–0.15 g/L; valine, 0.094 g/L; sodium selenate, 0.0000005–0.000060 g/L; magnesium sulfate, 0.05–0.2 g/L; potassium chloride, 0.15–0.45 g/L; sodium phosphate, 0.075–0.2 g/L; potassium nitrate, 0.00005–0.00009 g/L; calcium chloride, 0.08–0.25 g/L; sodium pyruvate 0.05–0.4 g/L; insulin, 0.05–2 g/L; hydrocortisone, 20–80 μg/L; linoleic acid, 1–100 mg/L; ethanolamine, 5–25 μg/L; sodium bicarbonate, 1–5 g/L; APO transferrin or ferric citrate, 1–10 mg/L; Pluronic F68, 0.2–2 g/L; sodium hydroxide, 0.3–0.9 g/L; mycophenolic acid, 0.1–2 mg/L; hypoxanthine, 2–5 mg/L; xanthine; 10–200 mg/L; sodium bicarbonate 1.5–4.5 g/L.

2. The chemically defined medium of claim **1** wherein the medium is suitable for growth of immortalized mammalian cells in culture to cell densities of at least 4.5×10^6 cells/ml.

3. The chemically defined medium of claim **1** wherein the immortalized mammalian cells are the myeloma cell line Sp2/0 or derivatives thereof.

* * * * *

EXHIBIT C

US006773600B2

(12) **United States Patent**
Rosenblatt et al.

(10) **Patent No.: US 6,773,600 B2**
(45) **Date of Patent: Aug. 10, 2004**

(54) **USE OF A CLATHRATE MODIFIER, TO PROMOTE PASSAGE OF PROTEINS DURING NANOFILTRATION**

(75) Inventors: **Barry P. Rosenblatt**, Morrisville, PA (US); **Richard C. Siegel**, Chester Springs, PA (US)

(73) Assignee: **Cantocor, Inc.**, Malvern, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/454,089**

(22) Filed: **Jun. 4, 2003**

(65) **Prior Publication Data**

US 2003/0230532 A1 Dec. 18, 2003

Related U.S. Application Data

(60) Provisional application No. 60/394,733, filed on Jun. 14, 2002.

(51) **Int. Cl.**[7] **B01D 61/20**; B01D 37/00; A61K 39/395; C07K 16/34

(52) **U.S. Cl.** **210/639**; 210/644; 210/651; 210/739; 210/743; 210/749; 424/176.1; 436/177; 436/178; 530/414

(58) **Field of Search** 210/639, 644, 210/650, 651, 652, 739, 743, 749, 767; 424/176.1; 436/177, 178; 530/387.1, 390.1, 414

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,789,482	A	12/1988	DiLeo et al.
6,096,872	A	8/2000	Van Holten et al.

FOREIGN PATENT DOCUMENTS

WO	WO 96/00237	A1	1/1996
WO	WO 96/37086	A1	8/1998

OTHER PUBLICATIONS

Dielo, Vacante, Deane, "Size Exclusion Removal of Model Mammalian Viruses Using a Unique Membrane System, Part 1: Membrane Qualification," Biologicals: 1993: 21, 275–286.

Dielo, Vacante, Deane, "Size Exclusion Removal of Model Mammalian Viruses Using a Unique Membrane System, Part II: Modular Qualification and Process Stimulation," Biologicals: 1993: 21, 287–296.

Dileo, Allegrezza, Jr, "Validatable virus removal from protein solutions," Nature 1991: 351, 420–421.

Nel, Oppenheim, Rodgers, Effects of Solution Properities on Solute and Permeate Flux in Bovine Serum Albumin–IgG Ultrafiltration. Biotechno. Prog. 1994: 10, 539–542.

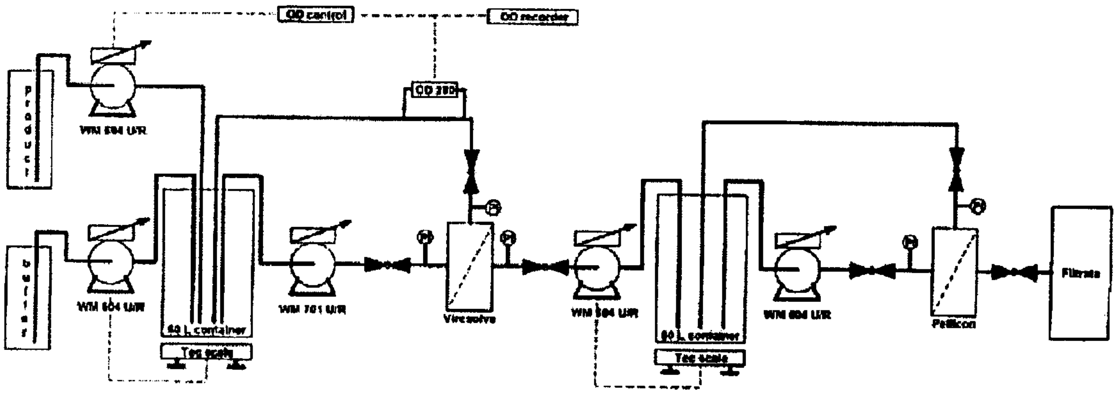
Primary Examiner—John Kim

(57) **ABSTRACT**

The invention relates to the field of protein purification and the recovery of large proteinaceous material through small, nanometer sized, pore exclusion filters for removal of contaminants such as viral pathogens.

7 Claims, 1 Drawing Sheet

Viresolve and Concentration / Diafiltration Process



Viresolve and Concentration / Diafiltration Process

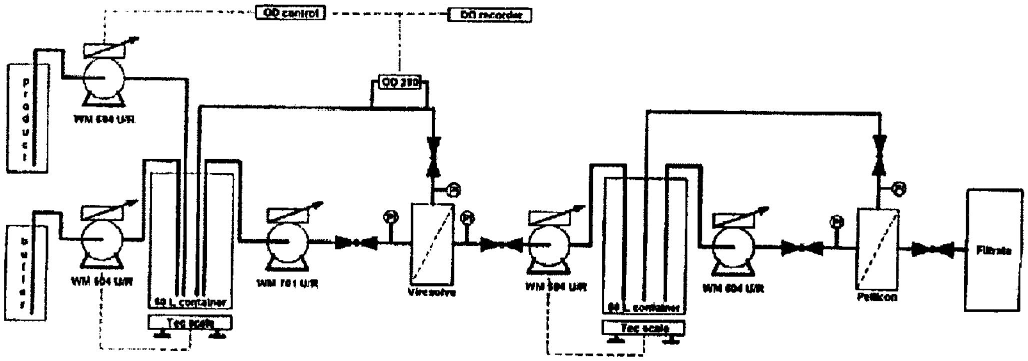

OD control
product
buffer
Tec scale
Tec scale
Viresolve
Pellicon
Filtrate

Figure 1

USE OF A CLATHRATE MODIFIER, TO PROMOTE PASSAGE OF PROTEINS DURING NANOFILTRATION

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 60/394,733, filed Jun. 14, 2002.

FIELD OF THE INVENTION

The invention relates to the field of protein purification and the recovery of large proteinaceous material through small, nanometer sized, pore exclusion filters for removal of contaminants such as viral pathogens. The invention relates to the use of additives to promote solubility of proteins in solutions being filtered for the purpose of removing pathogens, particularly viral pathogens, and has particular applicability to the purification of large proteinaceous biomolecules such as immunoglobulins.

BACKGROUND OF THE INVENTION

Liquid and gas separation processes are well known in the art. Most common separation processes involve a phase change, which increases the cost of the processes and often requires excessive temperature changes which can alter the product. Membrane separations, however, can achieve desired levels of separation without a change in the substances' phase. In essence, membrane separation selectively forces one or more substances through pores of a filter, leaving one or more larger substances behind. This process is often repeated with diminishing filter pore sizes until a satisfactory level of separation is achieved.

The use of nanofiltration to remove contaminants such as virus particles from parenteral protein products is based upon the ability of a filter of defined pore size to allow a soluble protein to pass through while denying passage of the larger viral particles (DiLeo, A J, et al, BioTechnology 1992, 10: 182,188.) Removal of virus from large biomolecules such as immunoglobulins (monoclonal or polyclonal antibodies), by size exclusion, is hindered by the difficulty of passing the large biomolecules through pore sizes of nanometer size, typically 12–15 nm. While a protein in solution, even one as large as an immunoglobulin, is expected to have a molecular radius much smaller than a viral particle, several factors can lead to an effective reduction in pore size and sieving coefficient. Some of these factors are due to interactions between the protein and the filter surface resulting in build up on the membrane surface known as a gelation or polarization layer. Other factors, such as protein self-association or aggregation, cause the protein to be trapped by the filter due to formation of masses too large to pass through the filter pores or that have surface characteristics that exhibit affinity for the membrane surface or pore surfaces causing them to adhere to the membrane instead of passing through.

International patent application, WO 9600237, describes methods for successful nanofiltration using pore sizes as small as 15 nm to filter purified proteins of molecular weight less than 150 kDa. WO 9600237 discloses the use of salt concentrations lying in the range from about 0.2 M up to saturation of the solution in virus-filtering of proteins, polysaccharides, and polypeptides to increase sieving coefficients. The advantage of the salt is stated by the applicants to be because the "protein contracts" and more easily passes through the filter. The use of a high salt content according to

this method is also suggested to enable the use of "dead-end" filtering with membranes having pore sizes of 5–30 nm. Dead-end filtering refers to the practice of using a single pump to force fluid through the membrane from the surface. Dead-end filtration is simpler and more cost effective than tangential filtering process wherein a first pump maintains constant flow rate at the surface of the membrane and a second pump draws the protein through the filter by creating a negative pressure (suction) at the back of the membrane.

U.S. Pat. No. 6,096,872 recognized the utility of adding surfactants along with high ionic strength buffering during nanofiltration to remove viruses from immunoglobulin containing solutions in order to reduce protein dimerization, trimerization and aggregation, the teachings of which are hereby incorporated herein by reference.

It is also generally known that in order to reduce the interaction of a substance with the membrane surface, the "zeta-" or "z-"potential of the membrane surface should not be electrically attractive to that substance and altering the charge properties of the membrane can minimize surface precipitation. For example, U.S. Pat. No. 6,177,011 teaches that the neutralization of surface charges measured as zeta potential can reduce surface adsorption of membrane-fouling substances during reverse osmosis filtration processes where the substance carries a charged group. Changes in pH and salt concentration are other means of altering the z-potential of both the solutes and the membrane surface. In some cases, however, the manipulation of the z-potential by the addition of salt is counter-productive, resulting in an increase in soluble aggregation and an increase in the hydrophobic character of the membrane surface which may promote interaction with hydrophobic protein regions. Pall, et al (Colloids and Surfaces 1 (1980), 235–256.), reported that the phenomenon of removal of particles smaller than the pores of a filter is due to adherence of the particles to the pore walls under conditions wherein the particles and the pore walls are oppositely charged or alternatively wherein the zeta potential of the particles and the pore walls of the membrane are both low. Zierdt (Applied and Environmental Microbiology, (1979) 38:1166–1172) attributed the aforementioned phenomenon to electrostatic forces. Furthermore, these modifications do not address the effects of molecular geometry or protein aggregation in solution on membrane filtration.

In addition to the considerations of buffer components and their concentrations, care must be take to maintain the protein to be filtered in a concentration appropriate to maintaining good flow and minimal transmembrane pressure across the filter. WO 9837086 teaches the addition of buffer to the retentate in order to maintain transmembrane pressure during tangential flow of a pretreatment step to remove proteins having a molecular weight greater than that of the product protein(s). WO 9837086 further notes that nanofiltration is limited to therapeutic proteins having a molecular weight up to 150 kDa. Immunoglobulin G molecules are composed of two heavy chains and two light chain polypetides all covalently linked and have an average molecular weight of about 180 kDa. U.S. Pat. No. 6,096,872 seeks to address the problem of how to filter viruses from IgG products by including a non-ionic excipient with relatively high (physiological which is about 300 mOsm) ionic strength buffer. The use of high ionic strength buffers, however, may lead to protein aggregation or create the problem of salt removal from the product formulation. U.S. Pat. No. 6,096,872 teaches and claims a second nanofiltration step to concentrate the immunoglobulin and collect it in a low ionic strength buffer.

These methods suffer from various disadvantages, particularly in their efficiency. It is therefore the object of the present invention to overcome the short-comings of the prior art, particularly in developing a system for efficiently filtering pathogenic viruses from immunoglobulin products, thereby providing virally cleared, pure immunoglobulin for injection.

The molecular configuration or size of a protein species has been predicted by changes in the partial specific volume and self-association of proteins. The change in partial specific volume of proteins so modified has been demonstrated by the independent measurements of sedimentation coefficients using analytical centrifugation. The method described herein uses the addition of a clathrate modifying substance to modify the molecular configuration of the protein to minimize specific volume and aggregation thereby enhancing passage of the protein through the membrane in a nanofiltration process.

SUMMARY OF THE INVENTION

The method of the invention maximizes protein passage during membrane filtration by using buffer additives aimed to increase the hydrophobicity of the membrane surface and decrease the hydrodynamic radius of the protein as well as reduce the tendency for the self-association of the protein desired to be filtered. The method of the invention first maximizes protein passage by decreasing the pH and the salt of the buffer which increases the hydrophobicity of the membrane surface and decreases the hydrodynamic radius of the protein. Secondly, a clathrate modifer is included in the buffer which modifier decreases the hydrodynamic radius of the protein while minimizing the tendencies for the protein to associate with either itself or the membrane filter. Thirdly, the process optionally includes continuous in-line monitoring of the filtration in order to maintain the above parameters of pH and clathrate modifier constant while maintaining low local levels of soluble protein. The use of the methods of the invention result in an increase in sieving coefficient and the ability to maintain reduced trans-membrane pressure during virus particle filtration. The process is applicable to the purification of any large proteinaceous biomolecule, particularly immunoglobulins. The immunoglobulins may be a monoclonal or polyclonal immunoglobulin.

The clathrate modifier is perferably a polyol sugar or sugar alcohol having from 4 to 8 hydroxyl groups. Examples of preferred polyols are sugars, including mono-saccharides and disaccharides preferably sucrose. The concentration of the polyol used as a clathrate modifier will generally be 5% w/v or greater. The use of sucrose causes a decrease in the size of the molecule and a reduction in the tendency for self-association of the protein desired to be freed from virus particles.

Thus, the invention contemplates a method for purifying a proteinacious material such as an immunoglobulin comprising the steps of:

(a) admixing the proteinaceous material with:
   (i) a low pH, low conductivity buffer solution formulated to reduce the pH between 5.0 and 6.0, and to achieve an ionic strength of less than 30 mS/cm;
   (ii) a non-ionic surfactant; and
   (iii) a clathrate modifier;
(b) performing nanofiltration on the proteinaceous material to obtain a purified material substantially free of viral particles.

Preferably, the clathrate modifier is a polyol sugar or sugar alcohol having from 4 to 8 hydroxyl groups.

The method of the invention may also include conducting an in-line pre-filtering step and monitoring the concentration of the material by installing an in-line concentration controlling monitor to maintain the parameters of pH, and protein concentration within pre-set ranges optimal for the material being purified.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Is a schematic representation of the vessels and monitoring equipment used in nanofiltration and the direction of fluid flow.

DETAILED DESCRIPTION

The instant invention uses a combination of selection of buffer, non-ionic surfactant and the use of a clathrate modifier as processing aids during viral reduction or viral clearances using size exclusion nanofiltration for purification of large proteinaceous biomolecules. The invention allows a small pore size exclusion nanofilter to be used with a globular protein molecule such as an immunoglobulin in a manner which allows for efficient flowthrough, minimal yield loss and no significant change in the immunoglobulin characterization aggregate level or stability.

Virus removed from the proteinaceous material by the nanofiltration method of the invention include all potential categories of virus, both enveloped (for example HIV, Hepatitis B) and non-enveloped (for example Hepatitis A, Parvovirus B19).

The advantages of the use of the processing aids and the method of the present invention include:

(1) the reduction of processing time and increased yield since the conditions employed increase the hydrophobicity of the membrane surface and reduce the specific volume and aggregation of the proteinaceous material;

(2) the ability to use smaller pore size nanofilters, thereby ensuring removal of smaller size viral particles;

(3) the process can be automated for continuous monitoring to allow for maximum efficiency and highest product yield per filter area;

(4) the essential characteristics of the proteinaceous material are unaffected by the process maintaining the integrity and quality of the end product.

In a broad sense, a clathrate is a molecular association in which the result may form a particle. Clathrates are included among those complexes in which one component (the host) forms a cavity or, in the case of a crystal, a crystal lattice containing spaces in the shape of long tunnels or channels in which molecular entities of a second chemical species (the guest) are located. There is no covalent bonding between guest and host, the attraction being generally due to van der Waals forces. If the spaces in the host lattice are enclosed on all sides so that the guest species is "trapped" as in a cage, such compounds are known as "clathrates" or "cage" compounds". van der Waals forces and hydrophobic interactions bind the guest to the host molecule in clathrates and inclusion compounds. Examples of hydrogen-bonded molecules that form clathrates are hydroquinone and water, and host molecules of inclusion compounds, urea or thiourea.

In the present case, the term "clathrate modifier" means a substance that is capable of modifying the clathrate structure of a protein in an aqueous environment and reducing its overall specific volume. Substance such as large globular proteins are good candidates for clathrate modifiers because of their capability of forming hydrogen bonds in an aqueous environment. The polyol clathrate modifier of the present invention, modifies the clathrate complex of the proteinaceous material thereby reducing its specific volume and allowing for a reduction in processing time and greater flowthrough in the nanofiltration process.

In this specification by "polyol sugars and sugar alcohols" is meant a group of polyols having from 4 to 8 hydroxyl groups. Examples of preferred polyols are sugars, including monosaccharides and disaccharides, and sugar alcohols as well as derivatives thereof having from 4 to 8 hydroxyl groups.

Examples of monosaccharides having 4 hydroxyl groups are arabinose, ribose and xylose. An example of a sugar alcohol having 4 hydroxyl groups is the sugar alcohol derived from erythrose, i.e. erythritol.

Examples of monosaccharides having 5 hydroxyl groups are galactose, fructose, glucose and sorbose. An example of a sugar alcohol having 5 hydroxyl groups is the sugar alcohol derived from xylose, i.e. xylitol.

Examples of sugar alcohols having 6 hydroxyl groups are those derived from glucose and sorbose as well as from the hydrolysis products of sucrose, e.g. sorbitol and mannitol. Examples of disaccharides are maltose, lactose and sucrose, the latter being preferred, all of which contain 8 hydroxyl groups.

The large proteinaceous material which may be processed in accordance with the present invention include large globular proteins such as immunoglobulins (for example IgG) and fragments thereof, blood coagulation factors, growth hormones, apolipoproteins, enzymes and similar protein biomolecules, whether naturally occurring or genetically engineered.

The term "z-potential," as used herein, means surface charge. The surface charge of a particle is sometimes referred to as its z-potential, a measurement of charge which falls off with distance. The z-potential is directly correlated with the polarity or net charge of a compound.

As used herein, the term "nanofiltration" refers to filtration using size exclusion means where the pore size is of nanometer size. In general, the pore size of the nanofiltering units, also referred to as UF filters, employed in the production of substantially pure, virus-free immunoglobulin products of the instant invention is less than about 30 nm, most preferably less than about 15 nm. However, any membrane having the filter cutoff rating sufficient to reduce or eliminate non-enveloped virus from a proteinaceous solution can be employed in the processing methods of the invention. For example, the VIRESOLVE® 180 SYSTEM Ultrafiltration System (Millipore Corporation, Bedford, Mass.) unit may be employed, such unit having a molecular weight pore size rating of less than about 180 KD molecular weight or about 12 nm.

The nonionic surfactant or detergents which may be used in the present invention include the nonionic polyoxyethylene detergents for example the polysorbates, TWEENS; vinyl polymers, PLURONICS; polyoxyethylene-polypropylene polymers or co-polymers; Brij, Sterox-AJ, and Tritons. Most preferred is polyoxyethylene sorbitan monooleate, polysorbate 80 (TWEEN 80).

The buffer employed in the invention is selected from any suitable low pH, low conductivity buffer such as phosphate buffers, citrate buffers, borate buffers, acetate buffers and glycine buffers at a pH of about 5.0. The buffer is employed to maintain the pH below 6 and reduce aggregation of the protein thereby allowing more efficient flow, through the nanofilter. Preferably a buffer with a low ionic strength of 50 mM±/−20% is employed, preferably a sodium acetate buffer, pH 5.0.

The method involves transferring the protein of interest into a low pH (pH 5.0–6.0), low conductivity buffer (10–20 mS/cm), containing a non-ionic detergent such as TWEEN 80 at a concentration of 0.01% and sucrose at a concentration of between 5 and 10% w/v. The tangential flow apparatus is in fluid communication with several other vessels: a product tank, a buffer tank, and a feed/recirculation tank equipped with an agitator. The relationship of these vessels and the fluid flow between is shown in FIG. **1**.

The protein concentration used in the processing of the instant invention will be in the range of about 0.1% to about 1% by weight. Up to about 1% can be used when the protein is monomeric or monoclonal. For immunoglobulins such as a chimeric monoclonal IgG1, the initial protein concentration used for processing is about 1 to 10 mg/ml.

During processing and filtration, the protein concentration is preferably monitored to maintain optimal levels. As shown in FIG. **1**, this can be accomplished by the installation of an in line concentration monitor. A dead-end prefilter may be placed in the line between the feed/recirculation tank and the UF filter. A UV monitor is placed in-line between the UF filter and recirculation tank, on the retentate line. to provide a feed-back to the feed and buffer addition tanks to allow maintenance of the target protein concentration. Adjustment of the prefiltered product containing solution is achieved by the addition of buffer into the feed/recirculation tank to achieve the desired pH, conductivity, detergent concentration, and sucrose concentration. FIG. **1** shows the fluid flow from the feed/recirculation tank. During the filtration, the concentration of the retentate is kept constant by the addition of buffer in order to minimize protein-protein interaction. In the example shown, this is accomplished by control of the pumps supplying the product into the recirculation tank. By increasing/decreasing the speed of the pump, the concentration can be kept within a narrow specified range. A load cell under the recirculation tank is used as an addition feedback to the buffer pump to avoid overflowing the tank.

During filtration, the transmembrane pressure is preferably in the range of 0.2 to about 2.0 bar, most preferably maintained at less then about 1.0 bar. The sieving coefficient will preferably be in the range of 75–95% with excursions no lower than 60%.

EXAMPLE

A working example of this invention is demonstrated in the production of a chimeric human/mouse IgG1. The protein, after elution from a cation exchange column at pH 5.0, is placed in the product tank. The buffer tank is filled with 50 mM Sodium acetate, 6% sucrose, 0.01% polysorbate (tween) 80. The protein and buffer are mixed to achieve a final protein concentration of 2.0 ∀0.2 mg/mL in the feed tank. The filtration is started with a cross flow rate of xx mL/min/cm^2 and a permeate rate of no greater than yy mL/min/cm^2. Transmembrane pressure and retentate concentration is monitored to ensure that the process remains within the prescribed limits. Once the product tank is empty, the filters are rinsed with 3× the hold-up volume of the system to maximize the yield.

What is claimed is:

1. A method for purifying a proteinacious material comprising the steps of:

(a) admixing the proteinaceous material with:

(i) a low pH, low conductivity buffer solution formulated to reduce the pH between 5.0 and 6.0, and to achieve an ionic strength of less than 30 mS/cm;

(ii) a non-ionic surfactant; and

(iii) a clathrate modifier;

(b) performing nanofiltration on the proteinaceous material to obtain a purified material substantially free of viral particles.

2. The method of claim **1** wherein the proteinaceous material is an immunoglobulin.

3. The method of claim **1** wherein the clathrate modifier is a polyol sugar or sugar alcohol having from 4 to 8 hydroxyl groups.

4. The method of claim **3** wherein the polyol is a monosaccharides or disaccharides.

5. The method of claim **4** wherein the polyol is sucrose.

6. The method of claim **1** wherein the concentration of the polyol used as a clathrate modifier is about 5% w/v or greater.

7. A method for purifying a proteinacious material comprising the steps of:

a) admixing the proteinaceous material with a buffer solution:

adjusting the pH and the ionic strength of the buffer such that the pH is 5.0–6.0 and the ionic strength is less than 30 mS/cm;

b) adding a surfactant to the buffer to minimize protein-protein and protein-membrane interactions,

c) adding a clathrate modifier to the buffer, which clathrate modifier

i) Reduces the hydrodynamic radius of the protein and

ii) Minimizes the self-association of the protein;

d) installing an in-line prefilter to the system;

e) installing an in-line concentration controlling monitor to the system; and

f) using information from the in-line concentration controlling monitor to maintain the buffer parameter of pH and protein concentration within the range of pH of 5.0–6.0 and the ionic strength is less than 30 mS/cm.

* * * * *

EXHIBIT D

SAMSUNG BIOEPIS CO., Ltd.
107, Cheomdan-daero, Yeonsu-gu, Incheon, 21987, Republic of Korea
TEL: 82-32-455-6013, FAX: 82-32-455-6499

SAMSUNG

May 26, 2016

Via DHL

General Counsel
Janssen Biotech, Inc.
800 Ridgeview Drive
Horsham, PA 19044

Re: Samsung Bioepis BLA 761054 for Biosimilar to Remicade® Infliximab (BLA 103772)
Notice of FDA Acceptance of 42 U.S.C. § 262(k) Application and
Notice of Commercial Marketing Pursuant to 42 U.S.C. § 262(l)(8)(A)

Dear Sir/Madam:

Samsung Bioepis Co., Ltd. ("Bioepis") hereby provides notice to Janssen Biotech, Inc. that Bioepis has submitted to the United States Food and Drug Administration ("FDA") a Biologics License Application ("BLA") pursuant to 42 U.S.C. § 262(k) in order to obtain approval to engage in the commercial manufacture, use, and/or sale of a biosimilar to Remicade® infliximab. The Bioepis BLA has been assigned BLA No. 761054 by the FDA.

On May 20, 2016, the FDA provided notice to Bioepis that Bioepis BLA No. 761054 is sufficiently complete to permit substantive review, such that Bioepis BLA No. 761054 has been accepted for review by the FDA. The reference product for Bioepis BLA No. 761054 is Remicade® infliximab, for which the sponsor is Janssen Biotech, Inc. The active ingredient, strength, and dosage form of Bioepis' proposed drug product is infliximab for injection, 100 mg per vial.

Bioepis will not provide Janssen Biotech, Inc. with a copy of BLA No. 761054 or any information that describes the process or processes used to manufacture the biological

Finally, Bioepis appoints the following counsel to receive correspondence and service on its behalf with respect to this matter at the address provided below, and consents to electronic service by email directed to and received by the following counsel:

John Kilyk, Jr.
Leydig, Voit & Mayer, Ltd.
Two Prudential Plaza, Suite 4900

EXHIBIT E

LAW OFFICES

LEYDIG, VOIT & MAYER, LTD.

A PROFESSIONAL CORPORATION

TWO PRUDENTIAL PLAZA, SUITE 4900
CHICAGO, ILLINOIS 60601-6745

(312) 616-5600
FACSIMILE: (312) 616-5700
WWW.LEYDIG.COM

JAMES B. MUSKAL
DENNIS R. SCHLEMMER
H. MICHAEL HARTMANN
BRUCE M. GAGALA
CHARLES H. MOTTIER
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DEREK A. LIGHTNER*‡
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MICHAEL J. SCHUBERT
JOSHUA D. BISHOP
STELLA M. BROWN
SARAH E. AAGAARD
KRISTINA L. SWANSON
ROBERT A. HESS*‡
MENGLU LIN*‡
NICOLE E. KOPINSKI
ENES OVCINA‡
HELENA O. BEREZOWSKYJ
SHOSHANA G. MARVIN*‡
TARA N. GOODARZI

WASHINGTON

LEYDIG, VOIT & MAYER
700 THIRTEENTH STREET, N.W., SUITE 300
WASHINGTON, D.C. 20005-3960
(202) 737-6770
FACSIMILE: (202) 737-6776

SAN FRANCISCO BAY AREA

LEYDIG, VOIT & MAYER, LLP
THE ATRIUM BUILDING
1981 NORTH BROADWAY, SUITE 310
WALNUT CREEK, CALIFORNIA 94596-5083
(925) 482-0100
FACSIMILE: (925) 482-0110

FRANKFURT AM MAIN

LEYDIG, VOIT & MAYER, GMBH
LIEBIGSTRASSE 51
60323 FRANKFURT AM MAIN, GERMANY
+49 (0)69 713 7798-0
FACSIMILE: +49 (0)69 713 7798-29

OF COUNSEL

GORDON R. COONS
JOHN W. KOZAK
BRETT A. HESTERBERG
JEFFREY A. WYAND*‡
LYNN A. SULLIVAN
JEFFREY B. BURGAN

PATENT AGENTS

KRISTEN J. HARRELL
RACHEL J. MEJDRICH
DEREK W. BARNETT
KATHLEEN M. HELM-BYCHOWSKI
FRANCIS J. KOSZYK
WEIDAN FAN
GUY R. WATKINS
IDONGESIT E. EBONG
STEVEN B. COURTRIGHT
BENJAMIN F. STRICK
MARIA RESTREPO-HARTWIG*

ALL RESIDENT IN CHICAGO OFFICE EXCEPT AS NOTED WITH ASTERISK*
ALL ATTORNEYS ARE LICENSED IN ILLINOIS EXCEPT AS NOTED WITH ‡

January 22, 2017

VIA EMAIL

Gregory L. Diskant
Patterson Belknap Webb & Tyler LLP
1133 Avenue of the Americas
New York, NY 10036-6710

Re: Samsung Bioepis BLA No. 761054

Dear Mr. Diskant:

We are in receipt of your letter dated January 12, 2017, with respect to the referenced matter.

Bioepis will not, and indeed cannot, commercially market the product that is the subject of the referenced BLA prior to FDA approval. In addition, Bioepis will not commence commercial marketing of the product that is the subject of the referenced BLA before the expiration of 180 days from the receipt by Janssen of a commercial marketing notice from Bioepis that is proper under applicable law.

As you noted in your letter, Janssen was provided with a commercial marketing notice of the product that is the subject of the referenced BLA by way of Bioepis' letter of May 26, 2016 (which was received by Janssen on May 27, 2016). While you assert that such a commercial marketing notice prior to FDA licensure is not an effective notice "[u]nder controlling law," we note that the legal effect of such a notice will be considered by the U.S. Supreme Court in the upcoming months. *See Amgen, Inc. v. Sandoz, Inc.*, 794 F.3d 1347 (Fed. Cir. 2015), *cert. granted*, 84 U.S.L.W. 3455 (U.S. Jan. 13, 2017) (No. 15-1039), *and cert. granted on Amgen's cross-petition*, 84 U.S.L.W. 3549 (U.S. Jan. 13, 2017) (No. 15-1195).

Bioepis will assert its rights in accordance with the decision of the U.S. Supreme Court and will provide Janssen with an additional commercial marketing notice at the time of FDA licensure if necessary.

Very truly yours,

LEYDIG, VOIT & MAYER, LTD.

By: John Kilyk, Jr.

John Kilyk, Jr.

JKJ/tim

EXHIBIT F

SAMSUNG BIOEPIS
107, Cheomdan-daero, Yeonsu-gu, Incheon 21987 Republic of Korea
TEL: +82-32-455-3114 FAX: +82-32-455-6199

SAMSUNG

April 21, 2017

Via DHL

General Counsel
Janssen Biotech, Inc.
800 Ridgeview Drive
Horsham, PA 19044

Re: Samsung Bioepis BLA 761054 for Biosimilar to Remicade® Infliximab (BLA 103772)
Notice of FDA Approval of 42 U.S.C. § 262(k) Application and
Additional Notice of Commercial Marketing Pursuant to 42 U.S.C. § 262(l)(8)(A)

Dear Sir/Madam:

By way of its letter dated May 26, 2016, Samsung Bioepis Co., Ltd. ("Bioepis") notified Janssen Biotech, Inc. that Bioepis had submitted to the United States Food and Drug Administration ("FDA") a Biologics License Application ("BLA") pursuant to 42 U.S.C. § 262(k) in order to obtain approval to engage in the commercial manufacture, use, and/or sale of a biosimilar to Remicade® infliximab.

The Bioepis BLA was assigned BLA No. 761054 by the FDA. The reference product for Bioepis BLA No. 761054 is Remicade® infliximab, for which the sponsor is Janssen Biotech, Inc. The active ingredient, strength, and dosage form of Bioepis's proposed drug product is infliximab for injection, 100 mg per vial.

In its letter dated May 26, 2016, Bioepis provided notice to Janssen Biotech, Inc. in accordance with 42 U.S.C. § 262(l)(8)(A) that Bioepis will commence commercial marketing of the biological product that is the subject of BLA No. 761054 as soon as possible under applicable law after the FDA's approval to do so but no earlier than 180 days from the receipt by Janssen Biotech, Inc. of Bioepis's commercial marketing notice. Janssen Biotech, Inc. received Bioepis's commercial marketing notice on May 26, 2016, which is more than 180 days ago.

On April 21, 2017, the FDA provided notice to Bioepis that BLA No. 761054 has been approved by the FDA.

Accordingly, Bioepis is authorized by the FDA to commercially market the biological product that is the subject of BLA No. 761054, and Bioepis will commercially market the biological product that is the subject of BLA No. 761054 as soon as possible under applicable law.

In the event that the aforementioned commercial marketing notice earlier provided by Bioepis to Janssen Biotech, Inc. is held to be void or otherwise ineffective, as stated in Mr. Kilyk's letter of January 22, 2017, to Mr. Diskant as counsel for Janssen Biotech, Inc., Bioepis hereby notifies Janssen Biotech, Inc. in accordance with 42 U.S.C. § 262(l)(8)(A) that Bioepis will commence commercial marketing of the biological product that is the subject of BLA No. 761054 as soon as possible but no earlier than 180 days from the receipt by Janssen Biotech, Inc. of Bioepis's commercial marketing notice.

SAMSUNG BIOEPIS
107, Cheomdan-daero, Yeonsu-gu, Incheon 21987 Republic of Korea
TEL: +82-32-455-3114 FAX: +82-32-455-6199

SAMSUNG

General Counsel
Janssen Biotech, Inc.
April 21, 2017
Page 2

Finally, Bioepis appoints the following counsel to receive correspondence and service on its behalf with respect to this matter at the address provided below, and consents to electronic service by email directed to and received by the following counsel:

John Kilyk, Jr.
Leydig, Voit & Mayer, Ltd.
Two Prudential Plaza, Suite 4900
180 North Stetson Avenue
Chicago, Illinois 60601-6745
telephone: + 1 312 616 5665
email: jkilyk@leydig.com

Samsung Bioepis Co., Ltd.

Sunghun Son
Head, Intellectual Property Group